Food and Chemical Toxicology

Maternal Vitamin D Supplementation inhibits Bisphenol A-induced proliferation of Th17 cells in adult offspring --Manuscript Draft--

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Abstract:	<p>Bisphenol A (BPA) exposure can increase the risk of immune-related diseases in later life. Vitamin D3 (Vit D3) has been shown to have multiple immunomodulatory actions and has been used to treat immune diseases. However, the potential beneficial effects of Vit D3 on BPA-induced adverse effects in the immune system have not explored. We hypothesize that VitD3 may ameliorate BPA-induced side effects in the immune system, even in offspring of VitD3-supplemented mothers. Here, we established our experimental model by exposing pregnant dams with 1000 nM BPA with or without VitD3 (0.25 µg/kg, 1 µg/kg and 4 µg/kg) treatment. We show that mother's exposure to BPA increases proliferation of the spleen T helper 17 (Th17) cells and serum protein level of IL-17 in the offspring; however, VitD3 supplementation in mothers dose-dependently ameliorated these BPA-induced side effects on the immune system in the offspring as evidenced by attenuated upregulation of Th17 proliferation, and RORyt, IL-17, IL-6, and IL-23 expressions in the offspring. Our data provide the first evidence that maternal VitD3 supplementation offers benefits to the offspring by attenuating BPA-induced side effects on the immune system through vitamin D receptor (VDR)-dependent regulation of transcription factors and cytokines, suggesting its translational potential.</p>
Response to Reviewers:	<p>Dear Editor,</p> <p>Thank you for considering our manuscript titled "Maternal Vitamin D Supplementation inhibits Bisphenol A-induced proliferation of Th17 cells in adult offspring" submitted to Food and Chemical Toxicology (#ID FCT-D-20-00494 R1) and giving us the opportunity to revise the paper. We appreciate your careful reading of our manuscript, kind comments and valuable suggestions very much. We have carefully considered the comments and revised the manuscript according to the revision comments. We believe that we have appropriately addressed the concerns raised and the quality of the revised manuscript has been significantly strengthened based on reviewers' suggestions.</p> <p>Below are our point-to-point responses to Reviewers' comments. The relevant changes have been made in the original manuscript according to the comments of Reviewer. We have highlighted the changes to our revised manuscript within the document in red. In addition, some grammar and spelling errors have also been corrected.</p> <p>Yours sincerely</p> <p>Tong Shen, Ph.D.</p>

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Comments from the Editor and/or Reviewers (if available):

Reviewer #1: None

Response to Reviewer: Thanks to you for your carefully reviewed and important comments all the time. We appreciate your careful reading of our manuscript, kind comments and valuable suggestions very much.

Reviewer #2: The authors still did not respond to the comment 3.

Comment 3 : Figure legends, including figure 3-7, what dose "#p < 0.5 compared with 1000nM BPA alone group" this mean? P < 0.5 means nothing. Does it mean P < 0.05? Also, there are many groups, the author can use different letter to express the different between each groups.

Response to point: Thank you for your kind comment. The typo error has been corrected. Based on your suggestion, we tried to use different letters to express the differences between each group. However, the relationships between each group were quite complicated. We have enclosed the results of RORyt mRNA expression of offspring at PND 21 and PND42 as an example. Comparison among the group means was made using one-way analysis of variance (ANOVA) at each time point followed by post hoc test and pairwise comparison [Tukey (2-sided)].

We enclosed the Table 1-4 and Figure 1 in the Detailed Response to Reviewers. As shown in Table 1-4, it is hard to use different letter to express the differences between each group. Our main aim was to explore whether maternal VitD3 supplementation could inhibit Th17 cell increase in offspring induced by perinatal environmental level BPA exposure in mice model and explored the potential molecular mechanisms of these action. Based on your suggestion, we changed our way to present the differences between the groups by using letters (See Figure 1 as an example) so as to be clear and easy to understand. We hope this was acceptable.

Reviewer #3: I have just a question:

The highlights should be revised. Each Highlight can be no more than 85 characters.

Response to point : Thanks for your important comments. We have revised our highlights accordingly. Under your important comments, this manuscript has become more rigorous in the continuous improvement. Once again, special thanks to you for your helpful comments and rigorous suggestions.

Response to the Reviewers

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Yours sincerely

Tong Shen, Ph.D.

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Table 1. Multiple Comparisons of RORyt mRNA expression at PND21

(I) Group	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Blank control	Vehicle control	-.01211	.06967	1.000	-.2141	.1899
	1000 nM BPA	-.67346*	.06967	.000	-.8754	-.4715
	1000nM BPA+0.25 µg/kg VitD3	-.40489*	.06967	.000	-.6069	-.2029
	1000nM BPA+1 µg/kg VitD3	-.25910*	.06967	.004	-.4611	-.0571
	1000nM BPA+4 µg/kg VitD3	-.05272	.06967	.974	-.2547	.1493
Vehicle control	Blank control	.01211	.06967	1.000	-.1899	.2141
	1000 nM BPA	-.66135*	.06967	.000	-.8633	-.4594
	1000nM BPA+0.25 µg/kg VitD3	-.39278*	.06967	.000	-.5948	-.1908
	1000nM BPA+1 µg/kg VitD3	-.24699*	.06967	.007	-.4490	-.0450
	1000nM BPA+4 µg/kg VitD3	-.04061	.06967	.992	-.2426	.1614
1000 nM BPA	Blank control	.67346*	.06967	.000	.4715	.8754
	Vehicle control	.66135*	.06967	.000	.4594	.8633
	1000nM BPA+0.25 µg/kg VitD3	.26857*	.06967	.003	.0666	.4705
	1000nM BPA+1 µg/kg VitD3	.41436*	.06967	.000	.2124	.6163
	1000nM BPA+4 µg/kg VitD3	.62073*	.06967	.000	.4188	.8227
1000nM BPA+0.25 µg/kg VitD3	Blank control	.40489*	.06967	.000	.2029	.6069
	Vehicle control	.39278*	.06967	.000	.1908	.5948
	1000 nM BPA	-.26857*	.06967	.003	-.4705	-.0666
	1000nM BPA+1 µg/kg VitD3	.14579	.06967	.299	-.0562	.3478
	1000nM BPA+4 µg/kg VitD3	.35217*	.06967	.000	.1502	.5541
1000nM BPA+1 µg/kg VitD3	Blank control	.25910*	.06967	.004	.0571	.4611
	Vehicle control	.24699*	.06967	.007	.0450	.4490

	1000 nM BPA	-.41436*	.06967	.000	-.6163	-.2124
	1000nM BPA+0.25 µg/kg VitD3	-.14579	.06967	.299	-.3478	.0562
	1000nM BPA+4 µg/kg VitD3	.20638*	.06967	.042	.0044	.4084
1000nM BPA+4 µg/kg VitD3	Blank control	.05272	.06967	.974	-.1493	.2547
	Vehicle control	.04061	.06967	.992	-.1614	.2426
	1000 nM BPA	-.62073*	.06967	.000	-.8227	-.4188
	1000nM BPA+0.25 µg/kg VitD3	-.35217*	.06967	.000	-.5541	-.1502
	1000nM BPA+1 µg/kg VitD3	-.20638*	.06967	.042	-.4084	-.0044

*. The mean difference is significant at the 0.05 level.

Table 2. Homogeneous Subsets of RORyt mRNA expression at PND21

Groups	N	Subset for alpha = 0.05		
		1	2	3
Blank control	20	1.3380		
Vehicle control	20	1.3501		
1000 nM BPA	20	1.3907		
1000nM BPA+0.25 µg/kg VitD3	20		1.5971	
1000nM BPA+1 µg/kg VitD3	20		1.7429	
1000nM BPA+4 µg/kg VitD3	20			2.0114
Sig.		.974	.299	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 20.000.

Table 3. Multiple Comparisons of RORyt mRNA expression at PND42

(I) Group	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Blank control	Vehicle control	-.01838	.06461	1.000	-.2057	.1689
	1000 nM BPA	-.47265*	.06461	.000	-.6599	-.2854
	1000nM BPA+0.25 µg/kg VitD3	-.30830*	.06461	.000	-.4956	-.1210
	1000nM BPA+1 µg/kg VitD3	-.21462*	.06461	.015	-.4019	-.0273

	1000nM BPA+4 µg/kg VitD3	-.07945	.06461	.821	-.2667	.1078
Vehicle control	Blank control	.01838	.06461	1.000	-.1689	.2057
	1000 nM BPA	-.45427*	.06461	.000	-.6416	-.2670
	1000nM BPA+0.25 µg/kg VitD3	-.28992*	.06461	.000	-.4772	-.1026
	1000nM BPA+1 µg/kg VitD3	-.19624*	.06461	.034	-.3835	-.0090
	1000nM BPA+4 µg/kg VitD3	-.06106	.06461	.934	-.2483	.1262
1000 nM BPA	Blank control	.47265*	.06461	.000	.2854	.6599
	Vehicle control	.45427*	.06461	.000	.2670	.6416
	1000nM BPA+0.25 µg/kg VitD3	.16435	.06461	.120	-.0229	.3516
	1000nM BPA+1 µg/kg VitD3	.25803*	.06461	.002	.0707	.4453
	1000nM BPA+4 µg/kg VitD3	.39321*	.06461	.000	.2059	.5805
1000nM BPA+0.25 µg/kg VitD3	Blank control	.30830*	.06461	.000	.1210	.4956
	Vehicle control	.28992*	.06461	.000	.1026	.4772
	1000 nM BPA	-.16435	.06461	.120	-.3516	.0229
	1000nM BPA+1 µg/kg VitD3	.09368	.06461	.696	-.0936	.2810
	1000nM BPA+4 µg/kg VitD3	.22885*	.06461	.007	.0416	.4161
1000nM BPA+1 µg/kg VitD3	Blank control	.21462*	.06461	.015	.0273	.4019
	Vehicle control	.19624*	.06461	.034	.0090	.3835
	1000 nM BPA	-.25803*	.06461	.002	-.4453	-.0707
	1000nM BPA+0.25 µg/kg VitD3	-.09368	.06461	.696	-.2810	.0936
	1000nM BPA+4 µg/kg VitD3	.13517	.06461	.299	-.0521	.3225

1000nM BPA+4 µg/kg VitD3	Blank control	.07945	.06461	.821	-.1078	.2667
	Vehicle control	.06106	.06461	.934	-.1262	.2483
	1000 nM BPA	-.39321*	.06461	.000	-.5805	-.2059
	1000nM BPA+0.25 µg/kg VitD3	-.22885*	.06461	.007	-.4161	-.0416
	1000nM BPA+1 µg/kg VitD3	-.13517	.06461	.299	-.3225	.0521

*. The mean difference is significant at the 0.05 level.

Table 4. Homogeneous Subsets of RORyt mRNA expression at PND42

Groups	N	Subset for alpha = 0.05			
		1	2	3	4
Blank control	20	1.3104			
Vehicle control	20	1.3288			
1000 nM BPA	20	1.3899	1.3899		
1000nM BPA+0.25 µg/kg VitD3	20		1.5251	1.5251	
1000nM BPA+1 µg/kg VitD3	20			1.6187	1.6187
1000nM BPA+4 µg/kg VitD3	20				1.7831
Sig.		.821	.299	.696	.120

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 20.000.

As shown in Table 1-4, it is hard to use different letter to express the differences between each group. Our main aim was to explore whether maternal VitD3 supplementation could inhibit Th17 cell increase in offspring induced by perinatal environmental level BPA exposure in mice model and explored the potential molecular mechanisms of these action. Based on your suggestion, we changed our way to present the differences between the groups by using letters (See Figure 1 as an example) so as to be clear and easy to understand. We hope this was acceptable.

Fig.1. Effects of maternal VitD3 supplementation on the upregulation of spleen ROR γ t mRNA expression in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly. ROR γ t mRNA expression of spleen was detected using RT-qPCR. the gadph was used as the internal standard. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: p < 0.05 versus the vehicle control group; b: p < 0.05 versus the 1000nM BPA alone group.

Reviewer #3: I have just a question:

The highlights should be revised. Each Highlight can be no more than 85 characters.

Response to point : Thanks for your important comments. We have revised our highlights accordingly. Under your important comments, this manuscript has become more rigorous in the continuous improvement. Once again, special thanks to you for your helpful comments and rigorous suggestions.

Maternal Vitamin D Supplementation inhibits Bisphenol A-induced proliferation of Th17 cells in adult offspring

Gengfu Wang^{a, #, &}, Yingpei Li^{b, #}, Yun Li^{b, #}, Jiaxiang Zhang^a, Chengfan Zhou^a,
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Running Title: VitD3 suppresses BPA-induced proliferation of Th17 cells

Highlights

1. Maternal VitD3 supplementation inhibits BPA-induced Th17 proliferation in offspring.
2. VitD3 in mother mice attenuates BPA-induced ROR γ t expression in offspring.
3. VitD3 in mother mice reduces BPA-induced IL-6 and IL-23 increase in offspring.
4. VitD3 inhibit Th17 increment was mediated by upregulated VDR expression.

Abstract

Bisphenol A (BPA) exposure can increase the risk of immune-related diseases in later life. Vitamin D3 (Vit D3) has been shown to have multiple immunomodulatory actions and has been used to treat immune diseases. However, the potential beneficial effects of Vit D3 on BPA-induced adverse effects in the immune system have not explored. We hypothesize that VitD3 may ameliorate BPA-induced side effects in the immune system, even in offspring of VitD3-supplemented mothers. Here, we established our experimental model by exposing pregnant dams with 1000 nM BPA with or without VitD3 (0.25 µg/kg, 1 µg/kg and 4 µg/kg) treatment. We show that mother's exposure to BPA increases proliferation of the spleen T helper 17 (Th17) cells and serum protein level of IL-17 in the offspring; however, VitD3 supplementation in mothers dose-dependently ameliorated these BPA-induced side effects on the immune system in the offspring as evidenced by attenuated upregulation of Th17 proliferation, and *RORγt*, IL-17, IL-6, and IL-23 expressions in the offspring. Our data provide the first evidence that maternal VitD3 supplementation offers benefits to the offspring by attenuating BPA-induced side effects on the immune system through vitamin D receptor (VDR)-dependent regulation of transcription factors and cytokines, suggesting its translational potential.

Key words: Vitamin D3; Bisphenol A; Th17 cells; VDR; *RORγt*; endocrine disrupting chemical

Introduction

Bisphenol A (BPA), a common endocrine disrupting environmental chemical (EDC), is widely used in the manufacture of daily-life materials, such as cans, water bottles, tooth-sealing materials and thermal papers. Humans are ubiquitously exposed to environmental related BPA through the use of products containing BPA in daily life (Vandenberg et al., 2019). It is known that exposure to BPA is associated with adverse effects on human health, especially prenatal and postnatal BPA exposure which increases the risk of immune-related diseases in later life, including obesity, diabetes, tumors and autoimmune diseases (Trasande et al., 2012; Jochmanova et al., 2015; Wassenaar et al., 2017; Rancière et al. 2019). Growing studies indicate that BPA exposure affects immune cells, including CD4⁺ T lymphocytes, macrophages and neutrophils and their development (Ma et al., 2019; Gao et al., 2020). T helper (Th)17 cells have been described as a unique subset of pro-inflammatory Th cells and play critical roles in inflammation and the pathophysiology of various immune-related diseases by producing cytokines, such as interleukin (IL)-17 and IL-21 (Beringer et al., 2016; McGeachy et al., 2019). Targeting the regulation of Th17 cell differentiation and proliferation is an effective way of preventing and controlling Th17 cell related diseases and inflammatory diseases. Our previous study has demonstrated that gestational and lactational exposure to environmental level BPA can increase the Th17 cells in the offspring mice mediated by specific alteration to their transcription factor and regulatory cytokines. This may represent a novel mechanism by which perinatal exposure to BPA promotes immune-related diseases in childhood and adulthood (Luo et al., 2016).

However, effective measures to prevent Th17 cell related hazards induced by BPA are very limited.

A study has found that postnatal BPA exposure increased urinary excretion of 1,25-dihydroxyvitamin D3 [1,25(OH)₂D3 or VitD3] and decreased its concentration in blood (Kim et al., 2019), which suggests that BPA exposure could perturb VitD3 metabolism and supplementation with VitD3 might be of benefit by attenuating the development of immune-related diseases caused by environmental BPA exposure. Studies show that VitD3 not only takes part in suppressing the proliferation of immune cells, but also regulates the production of effector cytokines (Di Rosa et al., 2011; Szymczak and Pawliczak, 2016). VitD3 modulates the expression of molecule through activating VitD receptor (VDR) then binding to VitD responsive elements of the target genes (Di Rosa et al., 2011; Szymczak and Pawliczak, 2016). Research has found that VitD3 have preventive and therapeutic effects on autoimmune diseases via inhibitory effects on Th17 effector responses in a VDR-dependent manner (Provvedini et al., 1983; Murdaca et al., 2019; Yamamoto & Jorgensen, 2019). However, the effect of VitD3 supplementation on Th17 cells by perinatal exposure to BPA has not been examined. In this study, we therefore investigated whether maternal VitD3 supplementation **could** inhibit Th17 cell increase in offspring induced by perinatal environmental level BPA exposure in mice model and explored the potential molecular mechanisms of these action.

2 Materials and methods

2.1 BPA, VitD3 and Vehicle

BPA (purity > 99%, Sinopharm Chemical Reagent Co, China) and solution preparations have been documented in our previous study (Luo et al., 2016). Briefly, BPA dosing solutions were prepared in the vehicle, 0.1% DMSO (Sigma-Aldrich, St. Louis, MO) in purified water. VitD3 ($\geq$ 98% purity) was purchased from Boston Biomedical, Inc. (Norwood, MA, US) and stock solutions of 1mM VitD3 were prepared in purified water with 0.1% DMSO and stored at -20°C .

2.2 Animals and treatments

All experiment procedures were done with approval of the Animal Care and Use Committee at Anhui Medical University. The animals were treated humanely and all efforts were made to minimize their suffering. Animals and BPA treatment have been described in our previous study (Luo et al., 2016). In that study, we have found that exposure of pregnant dams to 1000nM BPA has significantly increased the number of Th17 cells in the offspring's spleen. Therefore, we used 1000nM BPA as exposure dose to assess the effects of VitD3 on the Th17 cells in this study. The doses of VitD3 used referred to the recommended nutrient intake value for pregnant women (Chinese Nutrition Society, 2014). The dosage of 1 $\mu\text{g/kg}$ VitD3, corresponding to the half of the recommended nutrient intake value (10 $\mu\text{g/day}$) for pregnant women, was set as the medium-dose group. Then, 0.25 $\mu\text{g/kg}$ and 4 $\mu\text{g/kg}$ were used as the low-dose group and high-dose group, respectively. Briefly, 8-week-old ICR mice housed in prewashed polycarbonate cages and were mated after one week's acclimation. Pregnant dams were housed individually and randomly assigned to six groups (n=10 for each)

including blank control group (purified water), vehicle control group (0.1% DMSO), 1000 nM BPA group, 1000nM BPA+0.25 µg/kg VitD3 group, 1000nM BPA+1µg/kg VitD3 group, and 1000nM BPA+4µg/kg VitD3 group. Dams were exposed to BPA through drinking water from GD0 until weaning [postnatal day (PND) 21] and different volumes of VitD3 or vehicle were administered via intraperitoneal (i.p.) injection once daily at the same time. The volume of VitD3 or vehicle used was 1mL/100g body weight. At PND 21, pups were weaned and assigned to the same gender groups with free access to food and purified water without BPA. At PND21 and PND42, one male and one female offspring mouse from each litter were sampled randomly. Mice were anesthetized by carbon dioxide inhalation, blood was collected from ocular venous plexus, then mice were sacrificed by cervical dislocation, the spleen was collected.

2.3 Flow cytometry (FCM) analysis

Th17 cell numbers in offspring spleen were measured by FCM, according our previous study (Luo et al., 2016). Briefly, splenocyte suspensions were prepared and treated with 50 µg/L phorbol 12-myristate 13-aceate (PMA, eBioscience, San Diego, CA), 500µg/ml ionomycin (eBioscience) and 200 µM monensin (eBioscience) for 4 hours. Cell surface staining was conducted with phycoerythrin (PE)-conjugated antimouse-CD4 monoclonal antibody (Biolegend). Intracellular staining was performed using fluorescein isothiocyanate (FITC)-conjugated antimouse-IL-17 (Biolegend). Finally, the cells were sorted by FCM with a FACSScan (BD Bioscience) and data were analyzed using Cell Quest software.

2.4 Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNAs were extracted by using RNeasy Mini Kit (Qiagen), and reverse transcription were conducted by using Reverse Transcription Kit PrimeScript® RT Master Mix (Takara). qRT-PCR was performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using Real time-PCR kit SYBR® Premix Ex Taq™ II (Takara) (Luo et al., 2016). Relative quantification of mRNA was calculated using the standard $2^{-\Delta\Delta C_t}$ method with Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) serving as internal standard. The primers for *vdr* (Takara, Dalian, China) were: 5'-CCCCACCTATGCCGACTTC-3' (sense) and 5'-TGGAGAATAGCTCCCTGTACTTACG-3' (anti-sense); the primers for retinoic acid-related orphan receptoryt (*RORyt*) and *gapdh* were previously described (Luo et al., 2016).

2.5 Enzyme-linked immunosorbent assay (ELISA)

Whole blood was immediately centrifuged to obtain serum. Contents of IL-6, IL-23, IL-17, IL-21 and transforming growth factor- β (TGF- β) in serum were quantified by ELISA Kits from Rapidbio Co (RapidBio Laboratory, USA), as instructed by the manufacturer.

2.6 Western Blot Analysis

Proteins were extracted from spleen tissue with RIPA buffer containing protease inhibitors (Thermo Scientific). The protein concentration was measured using a Nanodrop 2000 (Thermo Scientific, Holtsville, USA). 20 μ g of protein was electrophoresed through 12.5% SDS polyacrylamide gel (PAGE), and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk for 1 h and next incubated with the primary antibody (anti-mouse VDR,

abcam, 1:1000) at 4°C overnight. Thereafter, the membranes were incubated with the secondary antibody, horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (1:1000) at room temperature for 2h. Results were detected with enhanced chemiluminescence (ECL) kit (Pierce Biotech). Lamin A/C antibody was used as the loading control. Images were analyzed by ImageJ software.

2.7 Statistical analysis

Data are expressed in graphs as mean $\pm$ SEM unless otherwise stated. Statistical analysis and graphic presentation of the results were performed using SPSS software 21.0 (IBM Corporation, New York). Comparison among the group means was made using one-way analysis of variance (ANOVA) at each time point followed by *post hoc* test and pairwise comparison [Tukey (2-sided)]. $P < 0.05$ was considered statistically significant.

Results

3.1 Maternal VitD3 supplementation and BPA exposure do not affect pregnancy outcomes and weight gain of the offspring

Daily water consumption of dams was (6.03 ± 0.98) ml/day and (9.10 ± 0.36) ml/day during pregnancy and lactation, respectively. No significant difference in daily maternal water consumption was observed among various groups during pregnancy and lactation (data not shown), neither was body weight gain in dams (Fig. 1). Perinatal VitD3 supplementation and 1000nM BPA exposure did not significantly influence birth weight, litter size, survival, and gender ratio of the offspring mice (Table 1). Further,

maternal VitD3 supplementation and perinatal environmental level BPA exposure did not significantly influence the average weights of pups at any time point (Fig. 2A). As shown in Fig. 2B, there were no significant differences in spleen coefficient of offspring mice following perinatal BPA exposure and maternal VitD3 supplementation compared to control counterparts on PND21 ($P = 0.965$) and PND42 ($P = 0.885$).

3.2 Spleen VDR expression is upregulated in the offspring with maternal VitD3 supplementation

As a steroid hormone, VitD3 is able to activate and bind to VDR and then induce the expression of VitD responsive genes (Christakos et al., 2016; Wei & Christakos, 2015). We first determined whether maternal VitD3 supplementation upregulated spleen *vdr* mRNA and VDR protein expression in the offspring following perinatal BPA exposure. As shown in Fig. 3, maternal 1000nM BPA exposure did not upregulate spleen *vdr* mRNA or VDR protein expression in the offspring on both PND21 and PND42, but maternal VitD3 supplementation increased spleen *vdr* mRNA (Fig. 3A) and VDR protein expression (Fig. 3B) in a dose-dependent manner in the offspring following perinatal 1000nM BPA exposure on both PND21 ($P < 0.001$) and PND42 ($P < 0.001$). Compared with 1000 nM BPA alone group, the significant difference in *vdr* mRNA expression was first seen at the VitD3 dose of 1 $\mu\text{g/kg}$ on both PND21 and PND42; VDR protein increase occurred at the VitD3 dose of 1 $\mu\text{g/kg}$ on PND21 and 4 $\mu\text{g/kg}$ on PND42, respectively. These data suggest that maternal VitD3 supplementation upregulated spleen VDR expression in the offspring following perinatal BPA exposure.

3.3 Maternal VitD3 supplementation suppresses the increment of spleen Th17 cells and the elevation of serum Th17 derived cytokines in the offspring induced by perinatal BPA exposure

We investigated the effects of maternal VitD3 supplementation on the increase in offspring spleen Th17 cell numbers and elevation of serum Th17 produced cytokines induced by perinatal BPA exposure. As shown in Fig.4, compared with vehicle control, perinatal 1000nM BPA exposure significantly increased spleen Th17 cells in the offspring, and maternal VitD3 supplementation resulted in a dose-dependent reduction of Th17 cells on both PND21 ($P<0.001$) and PND42 ($P<0.001$). Compared with 1000 nM BPA alone group, significant differences were observed at the VitD3 dose of 1 $\mu\text{g}/\text{kg}$ on PND21 and 0.25 $\mu\text{g}/\text{kg}$ on PND42, respectively.

Th17 play roles in immune pathogenesis mainly through production of proinflammatory cytokines IL-17 and IL-21 (Bettelli et al., 2008; Korn et al., 2009). Therefore, we then evaluated the effects of maternal VitD3 supplementation on elevations of serum IL-17 and IL-21 content in the offspring caused by perinatal BPA exposure. As shown in Fig. 5, maternal VitD3 supplementation decreased the elevation of serum IL-17 and IL-21 content in a dose-dependent manner on both PND21 ($P<0.001$) and PND42 ($P<0.001$). Compared with 1000 nM BPA alone group, significant differences in both IL-17 and IL-21 occurred at a VitD3 dose of 1 $\mu\text{g}/\text{kg}$ on PND21, and 4 $\mu\text{g}/\text{kg}$ on PND42, respectively. Correlation analysis shows that serum IL-17 and IL-21 contents were associated with spleen Th17 cell density in the offspring on both PND21(IL-17 and Th17, $r = 0.704$; $p < 0.001$, $n = 120$; IL-21 and Th17, $r = 0.658$, $p < 0.001$, $n = 120$) and PND42 (IL-17

and Th17, $r = 0.487$; $p < 0.001$, $n = 120$; IL-21 and Th17, $r = 0.526$, $p < 0.001$, $n = 120$).

These results indicate that maternal VitD3 supplementation inhibited the increase in spleen Th17 cells and secretion of Th17 cell derived cytokines in the offspring induced by perinatal BPA exposure.

3.4 Maternal VitD3 supplementation attenuates the upregulation of spleen ROR γ t mRNA expression in the offspring induced by perinatal BPA exposure

ROR γ t is not only a characteristic transcription factor of Th17 cells, but also an important regulator for Th17 cell differentiation and IL-17 expression (Ivanov et al., 2006; Korn et al., 2009). To better understand the mechanism underlying the action of VitD3 on the development of Th17 cells by perinatal exposure to BPA, we further examined whether maternal VitD3 supplementation affected spleen ROR γ t mRNA expression in the offspring. As shown in Fig.6, maternal VitD3 supplementation attenuated the upregulation of ROR γ t mRNA expression in a dose-response manner ($P = 0.004$). Compared with 1000 nM BPA alone group, significant statistical differences in ROR γ t mRNA expression were observed from a starting dose of VitD3 at 0.25 μ g/kg on PND21, and 1 μ g/kg on PND42, respectively. These data demonstrate that maternal VitD3 supplementation could counteract the upregulation of spleen ROR γ t mRNA expression in offspring induced by perinatal BPA exposure.

3.5 Maternal VitD3 supplementation reduces elevation of serum IL-6 and IL-23 in offspring induced by perinatal BPA exposure, but not TGF- β

In addition to transcription factor ROR γ t, cytokines TGF- β , IL-6 and IL-23 are also involved in the development of Th17 cells (Bettelli et al., 2008; Korn et al., 2009). We

therefore further explored the effects of maternal VitD3 supplementation on the elevation of serum IL-6, IL-23 and TGF- β in the offspring induced by perinatal BPA exposure. As shown in Fig. 7A and Fig. 7B, maternal VitD3 supplementation reduces the elevation of serum IL-6 and IL-23 in a dose-dependent manner on both PND21 (IL-6, $P<0.001$; IL-23, $P<0.001$) and PND42 (IL-6, $P=0.001$; IL-23, $P<0.001$), with similar dose-response as seen with Th17 cells. Maternal VitD3 supplementation did not cause significant change in TGF- β level regardless of dose and time (Fig. 7C).

4. Discussion

In this study, we explored the role of maternal VitD3 supplementation in the development of Th17 cells induced by perinatal exposure to environmental level BPA. We found that the expression of *vdr* mRNA and VDR protein was increased in spleen of the offspring induced by maternal VitD3 supplementation. Low dose of BPA induced upregulation of spleen Th17 cell proliferation and Th17 associated cytokines (i.e., IL-17 and IL-21). The fact that this lower than recommended dose can still affect autoimmune specific cell type - Th17 emphasizes its risk to the general population. Importantly, maternal supplementation with VitD3 suppresses the increase of spleen Th17 cell and production of Th17 cell function related cytokines IL-17 and IL-21 in the offspring induced by perinatal BPA exposure in a dose-dependent manner. Furthermore, we explored the mechanism underlying action of VitD3 on the development of Th17 cells by perinatal BPA exposure. Furthermore, we found that maternal VitD3 supplementation suppressed the upregulation of Th17 cell-

characteristic transcription factor ROR γ t expression; on the other hand, maternal VitD3 supplementation decreased the elevation of serum IL-6 and IL-23, which are Th17 cell differentiation related cytokines, in the offspring induced by perinatal BPA exposure. Our findings suggest that VitD3 has a repressive effect on BPA induced immune response via VDR activation, and describe a new concept regarding maternal VitD3 supplementation and the immune system development in the offspring.

VitD3, a biologically active form vitamin D, is known to have strong modulatory effects in the immune system through an action on VDR (Mora et al., 2008; Rosen et al., 2016; Yamamoto & Jorgensen, 2019). VDR belongs to nuclear receptor superfamily and acts as ligand-activated transcription factor. Complex of VitD3-VDR forms heterodimer with the retinoid receptor (RXR) and induces the expression of VitD responsive genes via binding of the heterodimer to VitD response elements (VDRE) in the promoter region of these genes (Christakos et al., 2016; Wei & Christakos, 2015). Chang et al. used VDR knockout mice and found that VDR signal in activated CD4⁺T cells was essential for down-regulating the development of Th17 cells (Chang J.H. et al., 2010). Our study **shows** that maternal VitD3 supplementation upregulated spleen *vdr* mRNA and VDR protein expression in the offspring mice. These results are in accordance with previous publications on the inhibitory effect of VitD3 on Th17 cells and IL-17 associated with VDR (Tang et al., 2009; Chang S.H. et al., 2010; Joshi et al., 2011; Sun et al., 2018;), but more importantly demonstrate that in vivo maternal administration of Vit D3 can suppress prenatal BPA exposure induced upregulation of Th17 cells and associated cytokines in the offspring.

Several studies have shown that VitD3 has preventive and therapeutic effects on autoimmune diseases by reducing the number of Th17 cells and suppressing the production of Th17 derived proinflammatory cytokines (Chang J.H. et al., 2010; Chang S. H. et al., 2010; da Costa et al., 2016; Fawaz et al., 2016; Marinho et al., 2016; Qiu et al., 2017; Sun et al., 2018; Jafarzadeh et al., 2018; Sheikh et al., 2018). Our findings indicate that maternal VitD3 supplementation may confer protective effect to the offspring. Specifically, this maternal VitD3 intervention suppresses perinatal BPA exposure-induced Th17 cell proliferation and serum IL-17 and IL-21 rise in the offspring. Furthermore, these changes in IL-17 and IL-21 are coincided with the change in the number of Th17 cells, supporting a causal relationship. Mechanistically, we found that maternal VitD3 supplementation inhibited the upregulation of spleen ROR γ t mRNA expression and decreased the elevation of serum IL-6 and IL-23 in offspring induced by perinatal BPA exposure in a dose-dependent manner. This finding was consistent with previous studies, which have reported that VitD3 can reduce ROR γ t mRNA expression (Tang et al., 2009; Joshi et al., 2011; Palmer et al., 2011) but demonstrates for the first time that such mode of action can take place through mother-fetus vertical route, providing important translational value. ROR γ t has been identified as a key transcriptional factor for Th17 cells (Ivanov et al., 2006; Park, et al., 2014), and IL-6, IL-23 and TGF- β are associated with differentiation of Th17 cells. Therefore, to a large extent, the inhibitory effect of maternal VitD3 supplementation on perinatal BPA exposure-induced Th17 cell increase in offspring mice may be attributable to the reduction of ROR γ t mRNA expression and decreases in IL-6 and IL-23. Interestingly,

maternal VitD3 supplementation did not significantly influence serum TGF- β level in offspring mice. Thus, these data suggest a ROR γ t-mediated but TGF- β independent pathway in the inhibitory effect of VitD3 on prenatal BPA exposure-induced proliferation of spleen Th17 cells in the offspring. Our previous studies revealed that TGF- β was not markedly altered by gestational and lactational BPA exposure (Luo et al., 2016), consistent the observation from the current study. Together with these results, we interpret that TGF- β is not involved in BPA prenatal exposure stimulated Th17 increase and the suppression of Th17 cell increase in the offspring by maternal VitD3 supplementation is mainly through inhibiting the increase of the other differentiation factors (i.e., IL-6) and the growth and stabilization factor (i.e. IL-23).

In present study, we demonstrate that maternal VitD3 supplementation at the doses of 1 μ g/kg and 0.25 μ g/kg can significantly suppress the increase of spleen Th17 cells in the offspring induced by BPA exposure at PND21 and PND42. This dose of 1 μ g/kg is corresponding to half of the recommended nutrient intake value (10 μ g/day) for pregnant women, suggesting relevance to human conditions. These results agree with earlier reports of the ability of 1,25(OH) $_2$ D3 to reduce inflammation (Qiu et al., 2017) and autoimmune diseases (Jafarzadeh et al., 2018; Ysmail-Dahlouk et al, 2016) in mice and also suggest that Vitamin D's ability to suppress autoimmune reactions is likely via an action on Th17 cells. Population studies have found that prenatal VitD supplement ($\leq$ 600 IU/day) can reduce the risk of offspring wheeze by age 3 years (Roth et al., 2017). Those findings suggest that even low dosage of maternal VitD3 supplement can have long-term positive effects on the development of allergic

diseases associated with immune reactions. Such long-term benefit may also be seen with prenatal BPA exposure. Given the fact that Th17 cells involved in inflammation and autoimmunity (Beringer et al., 2016; McGeachy et al., 2019), a major step forward from the present study is that VitD3 may have a positive effect in the suppression of BPA induced inflammation and autoimmunity. This finding also demonstrates that maternal VitD3 supplementation can down-regulate the inflammation caused by BPA exposure in the offspring. From public health point of view, VitD3 could be a beneficial measure to control the hazards of environmental level BPA exposure.

Interestingly, significant differences were observed at the VitD3 dose of 1 µg/kg on PND21 and 0.25 µg/kg on PND42, respectively for Th17 cells, whereas significant statistical differences in RORyt mRNA expression were observed from a starting dose of VitD3 at 0.25 µg/kg on PND21, and 1µg/kg on PND42, respectively. These different effective doses of VitD3 on Th17 cell number and on RORyt expression suggests that there are other mechanisms for the effects of VitD3 on Th17 cells in addition to an action on RORyt. Previous studies have found that interactions among the transcription factors runt-related transcription factor 1 (Runx1), RORyt and forkhead box protein P3 (Foxp3) can regulate the differentiation of IL-17 producing T cells (Zhang et al., 2008). Studies also show that 1,25(OH)₂D₃ can suppress Th 17 cells via upregulating Foxp3 and Runx1 (Fawaz et al., 2016; Joshi et al., 2011). Vit D3 can also suppress Th 17 cells via upregulating cytotoxic T-Lymphocyte-associated antigen 4 (CTLA-4) (Jeffery et al., 2015; Fawaz et al., 2016). Additionally, 1,25(OH)₂D₃ can inhibit Th17 cellular immune response via the non-transcriptional pathway that

overexpressed C/EBP homologous protein (CHOP) (Chang S.H. et al., 2010). A recent study shows that the Janus kinase/signal transducers and activators of transcription (JAK/STAT) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway genes and epigenetic mechanisms is involved in the effects of Vit D on Th 17 cells (Zeitelhofer et al., 2017). To date, the mechanisms of the effect of VitD3 on Th17 cells are not fully understood. Future studies are needed to better understand the ROR γ t-independent mechanisms underlying VitD3 effects on Th17 cells.

In summary, our data provide the first evidence for a repressive effect of maternal VitD3 supplementation on Th17 cell proliferation in offspring mice induced by perinatal environmental level BPA exposure and suggest that this effect is at least in part, due to its positive effect on the down-regulation of ROR γ t mRNA expression, the differentiation factors (i.e., IL-6) and the growth and stabilization factor (i.e. IL-23), mediated by VDR activation, which underscore the immunomodulatory effect of VitD. These results extend previous studies and identify a protective effect of VitD3 by suppressing the negative immune effects induced by BPA exposure. Importantly, our finding show that maternal VitD3 supplement may be of benefit to the normal development of immune system in the offspring. However, the detailed molecular mechanisms on how VitD3 acts on Th17 cell proliferation induced by BPA exposure and how these factors interact with each other remain to be clarified. It would be of interest to explore the potential of VitD3 as therapeutic targets for autoimmune diseases and prevention of immune damage due to environmental chemical exposure.

Conflict of interest statement

The authors declare that there are no actual or potential conflicts of interest.

Acknowledgments

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Legends

Fig. 1. Effects of perinatal VitD3 supplementation and BPA exposure on the average body weight of dams. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 µg/kg, 1 µg/kg or 4 µg/kg) was injected i.p.. Body weight was recorded every three days. Data are expressed as mean ± S.E.M. (n = 10).

Fig. 2. Effects of maternal VitD3 supplementation and perinatal BPA exposure on body weight and spleen coefficient of pups. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 µg/kg, 1 µg/kg or 4 µg/kg) was injected i.p.. Litter body weight of pups was recorded weekly, average body weight of pups (A) was then calculated. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly. Spleen weight was recorded and spleen coefficient (B) was then calculated. Data are expressed as mean ± S.E.M. (n = 20).

Fig.3. Effects of maternal VitD3 supplementation and perinatal BPA exposure on spleen *vdr* mRNA and VDR protein expression in offspring mice. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 µg/kg, 1 µg/kg or 4 µg/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly.

A. Relative *vdr* mRNA expression was quantified by qRT-PCR, the *gapdh* was used as

the internal standard. B. VDR protein expression was analyzed by Western blot, Lamin A/C served as the loading control. The results are expressed as mean $\pm$ S.E.M. (n = 8). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Fig.4. Effects of maternal VitD3 supplementation on increases in spleen Th17 cell frequency in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly, and spleen Th17 cell frequency was analyzed by FCM. A. Representative flow cytometry plots for CD4 and IL-17 staining of Th17 cell frequency in spleen. B. Quantification of the frequency of Th17 cells. Data are expressed as mean $\pm$ S.E.M. (n = 20). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Fig.5. Effects of maternal VitD3 supplementation on the elevation of serum IL-17 and IL-21 in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, blood from one male and one female offspring mouse from each litter were sampled randomly. Contents of IL-17 (A) and IL-21 (B) in serum from the offspring mice were measured

by ELISA. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Fig.6. Effects of maternal VitD3 supplementation on the upregulation of spleen *ROR γ t* mRNA expression in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly. *ROR γ t* mRNA expression of spleen was detected using RT-qPCR. the *gadph* was used as the internal standard. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Fig.7. Effects of maternal VitD3 supplementation on the elevation of serum IL-6, IL-23 and TNF- β in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, blood from one male and one female offspring mouse from each litter were sampled randomly. Contents of IL-6 (A), IL-23 (B) and TNF- β (C) in serum from the offspring mice were measured by ELISA. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Table. 1 Effect of maternal various doses VitD3 supplementation and 1000nM BPA exposure during gestation on birth outcomes

Group	n	litter size at birth	Average birth weight(g)	Gender ratio (M/F)	Survival ratio at PND21(%)
Blank Control	10	12.9±2.02	1.67 ±0.10	1.04±0.23	0.93±0.05
Vehicle Control	10	12.7±1.64	1.67±0.12	1.08±0.24	0.90±0.08
1000 nM BPA	10	13.2±3.22	1.67±0.09	1.07±0.26	0.92±0.09
1000nM BPA+0.25 µg/kg Vit D3	10	12.4±2.99	1.66±0.09	1.09±0.29	0.92±0.08
1000nM BPA+1 µg/kg Vit D3	10	13.0±2.21	1.67±0.10	1.06±0.25	0.93±0.07
1000nM BPA+4 µg/kg Vit D3	10	12.9±2.23	1.66±0.08	1.10±0.40	0.92±0.08
<i>F Value</i>		0.154	0.165	0.054	0.154
<i>P Value</i>		0.978	0.974	0.998	0.978

Note: The unit for the litter size was the total number of the offspring mice in that litter.

Statistical data are expressed as mean ± standard deviation.

Introduction

Bisphenol A (BPA), a common endocrine disrupting environmental chemical (EDC), is widely used in the manufacture of daily-life materials, such as cans, water bottles, tooth-sealing materials and thermal papers. Humans are ubiquitously exposed to environmental related BPA through the use of products containing BPA in daily life (Vandenberg et al., 2019). It is known that exposure to BPA is associated with adverse effects on human health, especially prenatal and postnatal BPA exposure which increases the risk of immune-related diseases in later life, including obesity, diabetes, tumors and autoimmune diseases (Trasande et al., 2012; Jochmanova et al., 2015; Wassenaar et al., 2017; Rancière et al. 2019). Growing studies indicate that BPA exposure affects immune cells, including CD4⁺ T lymphocytes, macrophages and neutrophils and their development (Ma et al., 2019; Gao et al., 2020). T helper (Th)17 cells have been described as a unique subset of pro-inflammatory Th cells and play critical roles in inflammation and the pathophysiology of various immune-related diseases by producing cytokines, such as interleukin (IL)-17 and IL-21 (Beringer et al., 2016; McGeachy et al., 2019). Targeting the regulation of Th17 cell differentiation and proliferation is an effective way of preventing and controlling Th17 cell related diseases and inflammatory diseases. Our previous study has demonstrated that gestational and lactational exposure to environmental level BPA can increase the Th17 cells in the offspring mice mediated by specific alteration to their transcription factor and regulatory cytokines. This may represent a novel mechanism by which perinatal exposure to BPA promotes immune-related diseases in childhood and adulthood (Luo et al., 2016).

However, effective measures to prevent Th17 cell related hazards induced by BPA are very limited.

A study has found that postnatal BPA exposure increased urinary excretion of 1,25-dihydroxyvitamin D3 [1,25(OH)₂D3 or VitD3] and decreased its concentration in blood (Kim et al., 2019), which suggests that BPA exposure could perturb VitD3 metabolism and supplementation with VitD3 might be of benefit by attenuating the development of immune-related diseases caused by environmental BPA exposure. Studies show that VitD3 not only takes part in suppressing the proliferation of immune cells, but also regulates the production of effector cytokines (Di Rosa et al., 2011; Szymczak and Pawliczak, 2016). VitD3 modulates the expression of molecule through activating VitD receptor (VDR) then binding to VitD responsive elements of the target genes (Di Rosa et al., 2011; Szymczak and Pawliczak, 2016). Research has found that VitD3 have preventive and therapeutic effects on autoimmune diseases via inhibitory effects on Th17 effector responses in a VDR-dependent manner (Provvedini et al., 1983; Murdaca et al., 2019; Yamamoto & Jorgensen, 2019). However, the effect of VitD3 supplementation on Th17 cells by perinatal exposure to BPA has not been examined. In this study, we therefore investigated whether maternal VitD3 supplementation could inhibit Th17 cell increase in offspring induced by perinatal environmental level BPA exposure in mice model and explored the potential molecular mechanisms of these action.

2 Materials and methods

2.1 BPA, VitD3 and Vehicle

BPA (purity > 99%, Sinopharm Chemical Reagent Co, China) and solution preparations have been documented in our previous study (Luo et al., 2016). Briefly, BPA dosing solutions were prepared in the vehicle, 0.1% DMSO (Sigma-Aldrich, St. Louis, MO) in purified water. VitD3 ($\geq$ 98% purity) was purchased from Boston Biomedical, Inc. (Norwood, MA, US) and stock solutions of 1mM VitD3 were prepared in purified water with 0.1% DMSO and stored at -20°C .

2.2 Animals and treatments

All experiment procedures were done with approval of the Animal Care and Use Committee at Anhui Medical University. The animals were treated humanely and all efforts were made to minimize their suffering. Animals and BPA treatment have been described in our previous study (Luo et al., 2016). In that study, we have found that exposure of pregnant dams to 1000nM BPA has significantly increased the number of Th17 cells in the offspring's spleen. Therefore, we used 1000nM BPA as exposure dose to assess the effects of VitD3 on the Th17 cells in this study. The doses of VitD3 used referred to the recommended nutrient intake value for pregnant women (Chinese Nutrition Society, 2014). The dosage of 1 $\mu\text{g}/\text{kg}$ VitD3, corresponding to the half of the recommended nutrient intake value (10 $\mu\text{g}/\text{day}$) for pregnant women, was set as the medium-dose group. Then, 0.25 $\mu\text{g}/\text{kg}$ and 4 $\mu\text{g}/\text{kg}$ were used as the low-dose group and high-dose group, respectively. Briefly, 8-week-old ICR mice housed in prewashed polycarbonate cages and were mated after one week's acclimation. Pregnant dams were housed individually and randomly assigned to six groups (n=10 for each)

including blank control group (purified water), vehicle control group (0.1% DMSO), 1000 nM BPA group, 1000nM BPA+0.25 µg/kg VitD3 group, 1000nM BPA+1µg/kg VitD3 group, and 1000nM BPA+4µg/kg VitD3 group. Dams were exposed to BPA through drinking water from GD0 until weaning [postnatal day (PND) 21] and different volumes of VitD3 or vehicle were administered via intraperitoneal (i.p.) injection once daily at the same time. The volume of VitD3 or vehicle used was 1mL/100g body weight. At PND 21, pups were weaned and assigned to the same gender groups with free access to food and purified water without BPA. At PND21 and PND42, one male and one female offspring mouse from each litter were sampled randomly. Mice were anesthetized by carbon dioxide inhalation, blood was collected from ocular venous plexus, then mice were sacrificed by cervical dislocation, the spleen was collected.

2.3 Flow cytometry (FCM) analysis

Th17 cell numbers in offspring spleen were measured by FCM, according our previous study (Luo et al., 2016). Briefly, splenocyte suspensions were prepared and treated with 50 µg/L phorbol 12-myristate 13-aceate (PMA, eBioscience, San Diego, CA), 500µg/ml ionomycin (eBioscience) and 200 µM monensin (eBioscience) for 4 hours. Cell surface staining was conducted with phycoerythrin (PE)-conjugated antimouse-CD4 monoclonal antibody (Biolegend). Intracellular staining was performed using fluorescein isothiocyanate (FITC)-conjugated antimouse-IL-17 (Biolegend). Finally, the cells were sorted by FCM with a FACSScan (BD Bioscience) and data were analyzed using Cell Quest software.

2.4 Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNAs were extracted by using RNeasy Mini Kit (Qiagen), and reverse transcription were conducted by using Reverse Transcription Kit PrimeScript® RT Master Mix (Takara). qRT-PCR was performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using Real time-PCR kit SYBR® Premix Ex Taq™ II (Takara) (Luo et al., 2016). Relative quantification of mRNA was calculated using the standard $2^{-\Delta\Delta C_t}$ method with Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) serving as internal standard. The primers for *vdr* (Takara, Dalian, China) were: 5'-CCCCACCTATGCCGACTTC-3' (sense) and 5'-TGGAGAATAGCTCCCTGTACTTACG-3' (anti-sense); the primers for retinoic acid-related orphan receptoryt (*RORyt*) and *gapdh* were previously described (Luo et al., 2016).

2.5 Enzyme-linked immunosorbent assay (ELISA)

Whole blood was immediately centrifuged to obtain serum. Contents of IL-6, IL-23, IL-17, IL-21 and transforming growth factor- β (TGF- β) in serum were quantified by ELISA Kits from Rapidbio Co (RapidBio Laboratory, USA), as instructed by the manufacturer.

2.6 Western Blot Analysis

Proteins were extracted from spleen tissue with RIPA buffer containing protease inhibitors (Thermo Scientific). The protein concentration was measured using a Nanodrop 2000 (Thermo Scientific, Holtsville, USA). 20 μ g of protein was electrophoresed through 12.5% SDS polyacrylamide gel (PAGE), and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk for 1 h and next incubated with the primary antibody (anti-mouse VDR,

abcam, 1:1000) at 4°C overnight. Thereafter, the membranes were incubated with the secondary antibody, horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (1:1000) at room temperature for 2h. Results were detected with enhanced chemiluminescence (ECL) kit (Pierce Biotech). Lamin A/C antibody was used as the loading control. Images were analyzed by ImageJ software.

2.7 Statistical analysis

Data are expressed in graphs as mean $\pm$ SEM unless otherwise stated. Statistical analysis and graphic presentation of the results were performed using SPSS software 21.0 (IBM Corporation, New York). Comparison among the group means was made using one-way analysis of variance (ANOVA) at each time point followed by *post hoc* test and pairwise comparison [Tukey (2-sided)]. $P < 0.05$ was considered statistically significant.

Results

3.1 Maternal VitD3 supplementation and BPA exposure do not affect pregnancy outcomes and weight gain of the offspring

Daily water consumption of dams was (6.03 ± 0.98) ml/day and (9.10 ± 0.36) ml/day during pregnancy and lactation, respectively. No significant difference in daily maternal water consumption was observed among various groups during pregnancy and lactation (data not shown), neither was body weight gain in dams (Fig. 1). Perinatal VitD3 supplementation and 1000nM BPA exposure did not significantly influence birth weight, litter size, survival, and gender ratio of the offspring mice (Table 1). Further,

maternal VitD3 supplementation and perinatal environmental level BPA exposure did not significantly influence the average weights of pups at any time point (Fig. 2A). As shown in Fig. 2B, there were no significant differences in spleen coefficient of offspring mice following perinatal BPA exposure and maternal VitD3 supplementation compared to control counterparts on PND21 ($P = 0.965$) and PND42 ($P = 0.885$).

3.2 Spleen VDR expression is upregulated in the offspring with maternal VitD3 supplementation

As a steroid hormone, VitD3 is able to activate and bind to VDR and then induce the expression of VitD responsive genes (Christakos et al., 2016; Wei & Christakos, 2015). We first determined whether maternal VitD3 supplementation upregulated spleen *vdr* mRNA and VDR protein expression in the offspring following perinatal BPA exposure. As shown in Fig. 3, maternal 1000nM BPA exposure did not upregulate spleen *vdr* mRNA or VDR protein expression in the offspring on both PND21 and PND42, but maternal VitD3 supplementation increased spleen *vdr* mRNA (Fig. 3A) and VDR protein expression (Fig. 3B) in a dose-dependent manner in the offspring following perinatal 1000nM BPA exposure on both PND21 ($P < 0.001$) and PND42 ($P < 0.001$). Compared with 1000 nM BPA alone group, the significant difference in *vdr* mRNA expression was first seen at the VitD3 dose of 1 $\mu\text{g}/\text{kg}$ on both PND21 and PND42; VDR protein increase occurred at the VitD3 dose of 1 $\mu\text{g}/\text{kg}$ on PND21 and 4 $\mu\text{g}/\text{kg}$ on PND42, respectively. These data suggest that maternal VitD3 supplementation upregulated spleen VDR expression in the offspring following perinatal BPA exposure.

3.3 Maternal VitD3 supplementation suppresses the increment of spleen Th17 cells and the elevation of serum Th17 derived cytokines in the offspring induced by perinatal BPA exposure

We investigated the effects of maternal VitD3 supplementation on the increase in offspring spleen Th17 cell numbers and elevation of serum Th17 produced cytokines induced by perinatal BPA exposure. As shown in Fig.4, compared with vehicle control, perinatal 1000nM BPA exposure significantly increased spleen Th17 cells in the offspring, and maternal VitD3 supplementation resulted in a dose-dependent reduction of Th17 cells on both PND21 ($P<0.001$) and PND42 ($P<0.001$). Compared with 1000 nM BPA alone group, significant differences were observed at the VitD3 dose of 1 $\mu\text{g}/\text{kg}$ on PND21 and 0.25 $\mu\text{g}/\text{kg}$ on PND42, respectively.

Th17 play roles in immune pathogenesis mainly through production of proinflammatory cytokines IL-17 and IL-21 (Bettelli et al., 2008; Korn et al., 2009). Therefore, we then evaluated the effects of maternal VitD3 supplementation on elevations of serum IL-17 and IL-21 content in the offspring caused by perinatal BPA exposure. As shown in Fig. 5, maternal VitD3 supplementation decreased the elevation of serum IL-17 and IL-21 content in a dose-dependent manner on both PND21 ($P<0.001$) and PND42 ($P<0.001$). Compared with 1000 nM BPA alone group, significant differences in both IL-17 and IL-21 occurred at a VitD3 dose of 1 $\mu\text{g}/\text{kg}$ on PND21, and 4 $\mu\text{g}/\text{kg}$ on PND42, respectively. Correlation analysis shows that serum IL-17 and IL-21 contents were associated with spleen Th17 cell density in the offspring on both PND21(IL-17 and Th17, $r = 0.704$; $p < 0.001$, $n = 120$; IL-21 and Th17, $r = 0.658$, $p < 0.001$, $n = 120$) and PND42 (IL-17

and Th17, $r = 0.487$; $p < 0.001$, $n = 120$; IL-21 and Th17, $r = 0.526$, $p < 0.001$, $n = 120$).

These results indicate that maternal VitD3 supplementation inhibited the increase in spleen Th17 cells and secretion of Th17 cell derived cytokines in the offspring induced by perinatal BPA exposure.

3.4 Maternal VitD3 supplementation attenuates the upregulation of spleen ROR γ t mRNA expression in the offspring induced by perinatal BPA exposure

ROR γ t is not only a characteristic transcription factor of Th17 cells, but also an important regulator for Th17 cell differentiation and IL-17 expression (Ivanov et al., 2006; Korn et al., 2009). To better understand the mechanism underlying the action of VitD3 on the development of Th17 cells by perinatal exposure to BPA, we further examined whether maternal VitD3 supplementation affected spleen ROR γ t mRNA expression in the offspring. As shown in Fig.6, maternal VitD3 supplementation attenuated the upregulation of ROR γ t mRNA expression in a dose-response manner ($P = 0.004$). Compared with 1000 nM BPA alone group, significant statistical differences in ROR γ t mRNA expression were observed from a starting dose of VitD3 at 0.25 μ g/kg on PND21, and 1 μ g/kg on PND42, respectively. These data demonstrate that maternal VitD3 supplementation could counteract the upregulation of spleen ROR γ t mRNA expression in offspring induced by perinatal BPA exposure.

3.5 Maternal VitD3 supplementation reduces elevation of serum IL-6 and IL-23 in offspring induced by perinatal BPA exposure, but not TGF- β

In addition to transcription factor ROR γ t, cytokines TGF- β , IL-6 and IL-23 are also involved in the development of Th17 cells (Bettelli et al., 2008; Korn et al., 2009). We

therefore further explored the effects of maternal VitD3 supplementation on the elevation of serum IL-6, IL-23 and TGF- β in the offspring induced by perinatal BPA exposure. As shown in Fig. 7A and Fig. 7B, maternal VitD3 supplementation reduces the elevation of serum IL-6 and IL-23 in a dose-dependent manner on both PND21 (IL-6, $P<0.001$; IL-23, $P<0.001$) and PND42 (IL-6, $P=0.001$; IL-23, $P<0.001$), with similar dose-response as seen with Th17 cells. Maternal VitD3 supplementation did not cause significant change in TGF- β level regardless of dose and time (Fig. 7C).

4. Discussion

In this study, we explored the role of maternal VitD3 supplementation in the development of Th17 cells induced by perinatal exposure to environmental level BPA. We found that the expression of *vdr* mRNA and VDR protein was increased in spleen of the offspring induced by maternal VitD3 supplementation. Low dose of BPA induced upregulation of spleen Th17 cell proliferation and Th17 associated cytokines (i.e., IL-17 and IL-21). The fact that this lower than recommended dose can still affect autoimmune specific cell type - Th17 emphasizes its risk to the general population. Importantly, maternal supplementation with VitD3 suppresses the increase of spleen Th17 cell and production of Th17 cell function related cytokines IL-17 and IL-21 in the offspring induced by perinatal BPA exposure in a dose-dependent manner. Furthermore, we explored the mechanism underlying action of VitD3 on the development of Th17 cells by perinatal BPA exposure. Furthermore, we found that maternal VitD3 supplementation suppressed the upregulation of Th17 cell-

characteristic transcription factor ROR γ t expression; on the other hand, maternal VitD3 supplementation decreased the elevation of serum IL-6 and IL-23, which are Th17 cell differentiation related cytokines, in the offspring induced by perinatal BPA exposure. Our findings suggest that VitD3 has a repressive effect on BPA induced immune response via VDR activation, and describe a new concept regarding maternal VitD3 supplementation and the immune system development in the offspring.

VitD3, a biologically active form vitamin D, is known to have strong modulatory effects in the immune system through an action on VDR (Mora et al., 2008; Rosen et al., 2016; Yamamoto & Jorgensen, 2019). VDR belongs to nuclear receptor superfamily and acts as ligand-activated transcription factor. Complex of VitD3-VDR forms heterodimer with the retinoid receptor (RXR) and induces the expression of VitD responsive genes via binding of the heterodimer to VitD response elements (VDRE) in the promoter region of these genes (Christakos et al., 2016; Wei & Christakos, 2015). Chang et al. used VDR knockout mice and found that VDR signal in activated CD4⁺T cells was essential for down-regulating the development of Th17 cells (Chang J.H. et al., 2010). Our study shows that maternal VitD3 supplementation upregulated spleen *vdr* mRNA and VDR protein expression in the offspring mice. These results are in accordance with previous publications on the inhibitory effect of VitD3 on Th17 cells and IL-17 associated with VDR (Tang et al., 2009; Chang S.H. et al., 2010; Joshi et al., 2011; Sun et al., 2018;), but more importantly demonstrate that in vivo maternal administration of Vit D3 can suppress prenatal BPA exposure induced upregulation of Th17 cells and associated cytokines in the offspring.

Several studies have shown that VitD3 has preventive and therapeutic effects on autoimmune diseases by reducing the number of Th17 cells and suppressing the production of Th17 derived proinflammatory cytokines (Chang J.H. et al., 2010; Chang S. H. et al., 2010; da Costa et al., 2016; Fawaz et al., 2016; Marinho et al., 2016; Qiu et al., 2017; Sun et al., 2018; Jafarzadeh et al., 2018; Sheikh et al., 2018). Our findings indicate that maternal VitD3 supplementation may confer protective effect to the offspring. Specifically, this maternal VitD3 intervention suppresses perinatal BPA exposure-induced Th17 cell proliferation and serum IL-17 and IL-21 rise in the offspring. Furthermore, these changes in IL-17 and IL-21 are coincided with the change in the number of Th17 cells, supporting a causal relationship. Mechanistically, we found that maternal VitD3 supplementation inhibited the upregulation of spleen ROR γ t mRNA expression and decreased the elevation of serum IL-6 and IL-23 in offspring induced by perinatal BPA exposure in a dose-dependent manner. This finding was consistent with previous studies, which have reported that VitD3 can reduce ROR γ t mRNA expression (Tang et al., 2009; Joshi et al., 2011; Palmer et al., 2011) but demonstrates for the first time that such mode of action can take place through mother-fetus vertical route, providing important translational value. ROR γ t has been identified as a key transcriptional factor for Th17 cells (Ivanov et al., 2006; Park, et al., 2014), and IL-6, IL-23 and TGF- β are associated with differentiation of Th17 cells. Therefore, to a large extent, the inhibitory effect of maternal VitD3 supplementation on perinatal BPA exposure-induced Th17 cell increase in offspring mice may be attributable to the reduction of ROR γ t mRNA expression and decreases in IL-6 and IL-23. Interestingly,

maternal VitD3 supplementation did not significantly influence serum TGF- β level in offspring mice. Thus, these data suggest a ROR γ t-mediated but TGF- β independent pathway in the inhibitory effect of VitD3 on prenatal BPA exposure-induced proliferation of spleen Th17 cells in the offspring. Our previous studies revealed that TGF- β was not markedly altered by gestational and lactational BPA exposure (Luo et al., 2016), consistent the observation from the current study. Together with these results, we interpret that TGF- β is not involved in BPA prenatal exposure stimulated Th17 increase and the suppression of Th17 cell increase in the offspring by maternal VitD3 supplementation is mainly through inhibiting the increase of the other differentiation factors (i.e., IL-6) and the growth and stabilization factor (i.e. IL-23).

In present study, we demonstrate that maternal VitD3 supplementation at the doses of 1 μ g/kg and 0.25 μ g/kg can significantly suppress the increase of spleen Th17 cells in the offspring induced by BPA exposure at PND21 and PND42. This dose of 1 μ g/kg is corresponding to half of the recommended nutrient intake value (10 μ g/day) for pregnant women, suggesting relevance to human conditions. These results agree with earlier reports of the ability of 1,25(OH) $_2$ D3 to reduce inflammation (Qiu et al., 2017) and autoimmune diseases (Jafarzadeh et al., 2018; Ysmail-Dahlouk et al, 2016) in mice and also suggest that Vitamin D's ability to suppress autoimmune reactions is likely via an action on Th17 cells. Population studies have found that prenatal VitD supplement ($\leq$ 600 IU/day) can reduce the risk of offspring wheeze by age 3 years (Roth et al., 2017). Those findings suggest that even low dosage of maternal VitD3 supplement can have long-term positive effects on the development of allergic

diseases associated with immune reactions. Such long-term benefit may also be seen with prenatal BPA exposure. Given the fact that Th17 cells involved in inflammation and autoimmunity (Beringer et al., 2016; McGeachy et al., 2019), a major step forward from the present study is that VitD3 may have a positive effect in the suppression of BPA induced inflammation and autoimmunity. This finding also demonstrates that maternal VitD3 supplementation can down-regulate the inflammation caused by BPA exposure in the offspring. From public health point of view, VitD3 could be a beneficial measure to control the hazards of environmental level BPA exposure.

Interestingly, significant differences were observed at the VitD3 dose of 1 µg/kg on PND21 and 0.25 µg/kg on PND42, respectively for Th17 cells, whereas significant statistical differences in RORyt mRNA expression were observed from a starting dose of VitD3 at 0.25 µg/kg on PND21, and 1µg/kg on PND42, respectively. These different effective doses of VitD3 on Th17 cell number and on RORyt expression suggests that there are other mechanisms for the effects of VitD3 on Th17 cells in addition to an action on RORyt. Previous studies have found that interactions among the transcription factors runt-related transcription factor 1 (Runx1), RORyt and forkhead box protein P3 (Foxp3) can regulate the differentiation of IL-17 producing T cells (Zhang et al., 2008). Studies also show that 1,25(OH)₂D₃ can suppress Th 17 cells via upregulating Foxp3 and Runx1 (Fawaz et al., 2016; Joshi et al., 2011). Vit D3 can also suppress Th 17 cells via upregulating cytotoxic T-Lymphocyte-associated antigen 4 (CTLA-4) (Jeffery et al., 2015; Fawaz et al., 2016). Additionally, 1,25(OH)₂D₃ can inhibit Th17 cellular immune response via the non-transcriptional pathway that

overexpressed C/EBP homologous protein (CHOP) (Chang S.H. et al., 2010). A recent study shows that the Janus kinase/signal transducers and activators of transcription (JAK/STAT) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway genes and epigenetic mechanisms is involved in the effects of Vit D on Th 17 cells (Zeitelhofer et al., 2017). To date, the mechanisms of the effect of VitD3 on Th17 cells are not fully understood. Future studies are needed to better understand the ROR γ t-independent mechanisms underlying VitD3 effects on Th17 cells.

In summary, our data provide the first evidence for a repressive effect of maternal VitD3 supplementation on Th17 cell proliferation in offspring mice induced by perinatal environmental level BPA exposure and suggest that this effect is at least in part, due to its positive effect on the down-regulation of ROR γ t mRNA expression, the differentiation factors (i.e., IL-6) and the growth and stabilization factor (i.e. IL-23), mediated by VDR activation, which underscore the immunomodulatory effect of VitD. These results extend previous studies and identify a protective effect of VitD3 by suppressing the negative immune effects induced by BPA exposure. Importantly, our finding show that maternal VitD3 supplement may be of benefit to the normal development of immune system in the offspring. However, the detailed molecular mechanisms on how VitD3 acts on Th17 cell proliferation induced by BPA exposure and how these factors interact with each other remain to be clarified. It would be of interest to explore the potential of VitD3 as therapeutic targets for autoimmune diseases and prevention of immune damage due to environmental chemical exposure.

Conflict of interest statement

The authors declare that there are no actual or potential conflicts of interest.

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Legends

Fig. 1. Effects of perinatal VitD3 supplementation and BPA exposure on the average body weight of dams. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 µg/kg, 1 µg/kg or 4 µg/kg) was injected i.p.. Body weight was recorded every three days. Data are expressed as mean ± S.E.M. (n = 10).

Fig. 2. Effects of maternal VitD3 supplementation and perinatal BPA exposure on body weight and spleen coefficient of pups. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 µg/kg, 1 µg/kg or 4 µg/kg) was injected i.p.. Litter body weight of pups was recorded weekly, average body weight of pups (A) was then calculated. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly. Spleen weight was recorded and spleen coefficient (B) was then calculated. Data are expressed as mean ± S.E.M. (n = 20).

Fig.3. Effects of maternal VitD3 supplementation and perinatal BPA exposure on spleen *vdr* mRNA and VDR protein expression in offspring mice. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 µg/kg, 1 µg/kg or 4 µg/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly.

A. Relative *vdr* mRNA expression was quantified by qRT-PCR, the *gapdh* was used as

the internal standard. B. VDR protein expression was analyzed by Western blot, Lamin A/C served as the loading control. The results are expressed as mean $\pm$ S.E.M. (n = 8). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Fig.4. Effects of maternal VitD3 supplementation on increases in spleen Th17 cell frequency in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly, and spleen Th17 cell frequency was analyzed by FCM. A. Representative flow cytometry plots for CD4 and IL-17 staining of Th17 cell frequency in spleen. B. Quantification of the frequency of Th17 cells. Data are expressed as mean $\pm$ S.E.M. (n = 20). a: $p < 0.05$ versus the vehicle control group; b: $p < 0.05$ versus the 1000nM BPA alone group.

Fig.5. Effects of maternal VitD3 supplementation on the elevation of serum IL-17 and IL-21 in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, blood from one male and one female offspring mouse from each litter were sampled randomly. Contents of IL-17 (A) and IL-21 (B) in serum from the offspring mice were measured

by ELISA. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: p < 0.05 versus the vehicle control group; b: p < 0.05 versus the 1000nM BPA alone group.

Fig.6. Effects of maternal VitD3 supplementation on the upregulation of spleen *ROR γ t* mRNA expression in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, spleen from one male and one female offspring mouse from each litter were sampled randomly. *ROR γ t* mRNA expression of spleen was detected using RT-qPCR. the *gadph* was used as the internal standard. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: p < 0.05 versus the vehicle control group; b: p < 0.05 versus the 1000nM BPA alone group.

Fig.7. Effects of maternal VitD3 supplementation on the elevation of serum IL-6, IL-23 and TNF- β in offspring mice induced by perinatal BPA exposure. Pregnant dams were exposed to 1000nM BPA via drinking water from GD0 to PND21, at the same time VitD3 (0.25 μ g/kg, 1 μ g/kg or 4 μ g/kg) was injected i.p.. At PND21 and PND42, blood from one male and one female offspring mouse from each litter were sampled randomly. Contents of IL-6 (A), IL-23 (B) and TNF- β (C) in serum from the offspring mice were measured by ELISA. The results are expressed as mean $\pm$ S.E.M. (n = 20). a: p < 0.05 versus the vehicle control group; b: p < 0.05 versus the 1000nM BPA alone group.

Table. 1 Effect of maternal various doses VitD3 supplementation and 1000nM BPA exposure during gestation on birth outcomes

Group	n	litter size at birth	Average birth weight(g)	Gender ratio (M/F)	Survival ratio at PND21(%)
Blank Control	10	12.9±2.02	1.67 ±0.10	1.04±0.23	0.93±0.05
Vehicle Control	10	12.7±1.64	1.67±0.12	1.08±0.24	0.90±0.08
1000 nM BPA	10	13.2±3.22	1.67±0.09	1.07±0.26	0.92±0.09
1000nM BPA+0.25 µg/kg Vit D3	10	12.4±2.99	1.66±0.09	1.09±0.29	0.92±0.08
1000nM BPA+1 µg/kg Vit D3	10	13.0±2.21	1.67±0.10	1.06±0.25	0.93±0.07
1000nM BPA+4 µg/kg Vit D3	10	12.9±2.23	1.66±0.08	1.10±0.40	0.92±0.08
<i>F Value</i>		0.154	0.165	0.054	0.154
<i>P Value</i>		0.978	0.974	0.998	0.978

Note: The unit for the litter size was the total number of the offspring mice in that litter.

Statistical data are expressed as mean ± standard deviation.

Fig.1

Fig. 2

Fig.3

A

B

Fig.4

Fig.5

Fig.6

Fig.7

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.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Credit Author Statement

Wang G.F.: Writing- Original draft preparation and Visualization, Li Y.P.: Investigation, Li Y.: Investigation, Zhang J.X., Data curation and Software, Zhou C.F.: Methodology and Validation, Wu C.H.: Writing- Reviewing and Editing and Funding acquisition , Zhu Q.X.: Conceptualization and Supervision, Shen T.: Conceptualization, Funding acquisition and Project administration.