Co-exposure to fluoride and arsenic disrupts intestinal flora balance and induces testicular autophagy in offspring rats

Penghui Liu a,1, Ran Li b,c,1, Xiaolin Tian a,d, Yannan Zhao a, Meng Li a, Meng Wang a, Xiaodong Ying a, Jiyu Yuan a, Jiaxin Xie a, Xiaoting Yan c, Yi Lyu a, Cailing Wei a, Yulan Qiu a, Fengjie Tian a, Qian Zhao a, Xiaoyan Yan a,*,

a School of Public Health, Shanxi Medical University, Taiyuan, Shanxi 030001, China
b Shanxi Medical University School and Hospital of Stomatology, Taiyuan 030001, China
c Shanxi Province Key Laboratory of Oral Diseases Prevention and New Materials, Taiyuan, Shanxi 030001, China
d Shanxi Key Laboratory of Ecological Animal Science and Environmental Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, China
e Shanxi Medical University School and Hospital of Stomatology, Taiyuan 030001, China

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ABSTRACT

While numerous studies have shown that fluoride or arsenic exposure may damage the reproductive system, there are few reports of co-exposure to fluoride and arsenic. In addition, the literature on autophagy and intestinal flora composition in reproductive toxicity studies of co-exposure to fluoride and arsenic is insufficient. In this study, we developed a rat model of fluoride and arsenic exposure via drinking water from pre-pregnancy to 90 days postnatal. Sprague-Dawley rats were randomly divided into sterile water control group, fluoride group (100 mg/L NaF), arsenic group (50 mg/L NaAsO2) and combined exposure group (100 mg/L NaF+50 mg/L NaAsO2). Our results showed that fluoride and arsenic exposure caused a reduction in testicular weight and significant pathological damage to tissue. We found that the levels of follicle-stimulating hormone, luteinizing hormone, and testosterone were reduced to varying degrees. Meanwhile experiments showed that fluoride and arsenic exposure can modulate autophagic flux, causing increased levels of Beclin1 and LC3 expression and decreased p62 expression. Analogously, by performing 16S sequencing of rat feces, we found 24 enterobacterial genera that differed significantly among the groups. Furthermore, the flora associated with testicular injury were identified by correlation analysis of hormonal indices and autophagy alterations with intestinal flora composition at the genus level, respectively. In summary, our study shows that fluoride and arsenic co-exposure alters autophagic flux in the testis, causes testicular injury, and reveals an association between altered intestinal flora composition and testicular injury.

1. Introduction

Increasing investigations have shown that concurrent fluoride (F) and arsenic (As) contamination in drinking water has become a common global health problem. Epidemiological studies have shown high levels of F and As in groundwater in many parts of the world, such as China (Wang et al., 2012), Mexico (Sarirana-Ruiz et al., 2017) and India (Adimalla, 2019). An epidemiological study from Mexico revealed that co-exposure of F and As altered arsenic metabolism in Mexican adults, and there was an interaction between F and As (Jiménez-Córdova et al., 2019). Correspondingly, experimental studies have demonstrated that simultaneous exposure to F and As produces an antagonistic effect, thereby reducing their toxic effects (Guo et al., 2020). Furthermore, cumulative evidence suggests that Long-term high concentrations of F or As intake can cause damage to human and animal health, impairing the normal physiological functions of the heart (Miltonprabu and Thangapandiyan, 2015), kidney (Chattopadhyay et al., 2011), liver (Zhou et al., 2015) and other organs (Ma et al., 2012). Of note, excessive F or As can damage humans and animals reproductive systems (Long et al., 2009). Correct reproductive system development is arguably the most important feature in the life history of an organism (Pask, 2016). Testes are an important part of the male reproductive system, which can

* Corresponding author.
E-mail address: yanxiaoyan@sxmu.edu.cn (X. Yan).
1 Penghui Liu and Ran Li are equal to this work.

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produce and discharge sperm and secrete androgens. Previous studies have found that the reproductive toxicity of F or As can cause testicular damage (Zhang et al., 2013; Souza et al., 2019). For example, F can affect the blood-testis barrier (Zhang et al., 2016a), destroy the testicular tissue structure (Wei et al., 2016), reduce the quality and quantity of sperm (Feng et al., 2015), and affect the level of testosterone and other reproductive hormones in the serum (Niu et al., 2015). As also can affect serum levels of testosterone and sperm formation (Li et al., 2018). Chronic exposure to As can induce hypogonadism, leading to reduced sperm count and testicular damage (Ahangarpour et al., 2019). High levels of As exposure resulted in massive germ cell degeneration and alterations in follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) levels have also been reported (Zubair et al., 2017). However, the molecular mechanisms underlying these effects are still not fully elucidated.

Autophagy is an important lysosomal catabolic mechanism involved in the degradation of cellular proteins and organelles (Mizushima and Komatsu, 2011). Autophagy-related proteins, including Beclin1, LC3 and p62, play an important role in inducing autophagy (Levine and Kroemer, 2008). Our previous studies showed that co-exposure of F and As to the kidney could elevate the expression levels of Beclin1 and LC3 and induce the formation of autophagic microsomes (Tian et al., 2020). Numerous studies have demonstrated that exposure to F or As can cause alterations in autophagic flow in the nervous system (Manthari et al., 2018; Zhou et al., 2019). In addition, there is substantial evidence that excessive F exposure increases the number of autophagosomes and expression levels of the autophagic degradation substrate p62 in the testis, suggesting impaired autophagic degradation (Zhang et al., 2016b, 2017). However, there are still many gaps in the study of the effects of F and As co-exposure on autophagy in the testis.

Recent studies have shown that chemical toxicant exposure can cause damage to the intestinal barrier, resulting in altered intestinal flora (Rosenfeld, 2017; Liu et al., 2019). As we known stable intestinal flora plays an important role in maintaining the nutritional and metabolic functions of the body, protecting the integrity of the intestinal mucosa and regulating the immune response. Studies have shown that excessive F exposure can severely damage the rectal structure of mice, inhibit the proliferation of rectal epithelial cells, disturb the intestinal microflora, and cause intestinal dysbiosis (Wang et al., 2020). Even excessive F exposure can alter the intestinal flora profile of broilers and silkworms (Li et al., 2015; Luo et al., 2016). In addition, it has been shown that disruption of the composition of the intestinal flora can alter the transformation and hepatotoxicity of As in mice (Chi et al., 2019). Studies have reported that altered intestinal flora is associated with the reproductive system, such as the pathogenesis of polycystic ovary syndrome, which is linked to intestinal dysbiosis (Guo et al., 2016). However, the link between altered intestinal flora and testicular damage is unknown.

In summary, based on the role of F or As exposure induced autophagy in testicular injury and the effects of altered intestinal flora composition, we aimed to investigate the toxic effects of F and As co-exposure on the testis of offspring rats, with special attention to the role of regulated autophagic flux in testicular injury. We exposed offspring rats to F and As (in utero until 90 days postnatal) via drinking water, confirming the role of altered autophagic flux in testicular injury. Further 16S sequencing of feces from offspring rats revealed a link between changes in intestinal flora composition and testicular damage. Finally, we used factor analysis to identify a possible antagonistic interaction between F and As.

2. Materials and methods

2.1. Materials

Sodium fluoride (NaF) and sodium arsenite (NaAsO2) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals and treatment

Healthy adult Sprague-Dawley rats (12 females, 4 males) were provided by the Experimental Animal Center of Shanxi Medical University and were acclimatized for 2 weeks prior to the experiment. They were randomly divided into 4 groups: control group (sterile water), fluoride exposure group (100 mg/L NaF), arsenic exposure group (50 mg/L NaAsO2) and combined exposure group (100 mg/L NaF + 50 mg/L NaAsO2). After 10 days of exposure via drinking water, in-cage mating was performed at a 3:1 ratio, and the occurrence of vaginal embolism in female rats was defined as day 0 of gestation. On postnatal day 21, six healthy male pups were randomly selected from each group, as were mothers treated at the beginning of exposure, until 90 days after birth. F and/or As exposure was continuous throughout maternal pregnancy and from 21 days to 90 days postnatal in the pups. Male offspring of the rats were selected as experimental animals. The experimental protocol has been approved by the Experimental Animal Center and Ethics Committee of Shanxi Medical University.

2.3. Organ coefficient

After the animals were killed, the testes were immediately separated and weighed, and the testicular viscera coefficients of each group of experimental animals were calculated as follows:

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\text{Organ coefficient} = \frac{[\text{Organ wet weight (g)/Body weight (g)]}}{100}\%.
\]

2.4. Morphological examination

In brief, the testicular tissue was separated and made into paraffin sections, which were then baked at 60 °C for 1–2 h in the oven. Paraffin sections were dewaxed in conventional xylene and gradient alcohol, dyed with hematoxylin-eosin dye (HE), dehydrated and sealed with neutral gum (Feldman and Wolfe, 2014). Histological changes were observed under microscope.

2.5. Detection of serum reproductive hormone levels

The whole blood of the rats was separated to obtain the serum, which was stored in the refrigerator at −80 °C for analysis. The serum levels of testosterone (T), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in rats were determined using the corresponding enzyme-linked immunoassay (ELISA) kit (AndyGene, Beijing, China). The test steps are performed in strict accordance with the kit instructions.

2.6. Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from testicular tissue samples (30 mg tissue; n = 6/group) using TRIzol reagent (Takara, Dalian, China). The concentration and purity were determined by RNA purity analyzer (BioTek, Winooski, VT, USA). cDNA was obtained by reverse transcription using the PrimeScript RT reagent kit (Takara, Dalian, China). qPCR was performed using the LineGene 9660 fluorescence quantifier (Bori, Winooski, VT, USA). The primer sequences were designed according to mRNA sequences retrieved from PubMed and synthesized by Takara company (Table S1). The β-actin gene serves as an internal reference. The PCR reaction system consisted of 20 μL (10 μL SYBR® Premix Ex Taq™II, 8 μL primer and 2 μL cDNA). PCR amplification conditions were: pre-denaturation for 30 s at 95 °C, polymerase chain reaction (PCR) at 95 °C for 40 cycles of 5 s, 60 °C for 30 s, 72 °C for 30 s. The data was analyzed using the 2^ΔΔCt method.
2.7. Total protein extraction and Western blot analysis

The total protein of testicular tissue was extracted with RIPA lysis buffer (100:1 containing PMSF) and centrifuged at 12,000 g for 10 min at 4 °C. Protein concentrations were determined using the BCA protein kit (Kengene, nanjing, China). The proteins were separated by 10% SDS polyacrylamide gel electrophoresis. The target band was then transferred to the nitrocellulose membrane by running at 80 V for 90 min. Next, seal the membrane with 5% BSA at room temperature for 2 h. The membranes were then treated with LC3 (1:1000, Wuhan, China), p62 (1:1000, Abcam, USA), Beclin1 (1:2000, Abcam, USA) and β-actin (1:1000, Abcam, USA) at 4 °C overnight. The membranes were then rinsed with TBST for 3 times (10 min each) and incubated at room temperature with HRP-conjugated secondary antibodies (1:4000, Absin Bioscience Inc, China) for 1 h. Finally, the target protein bands were observed by enhanced chemiluminescence (ECL) solution.

2.8. Monodansylcadaverine (MDC) Staining for autophagic vacuoles

Paraffin sections were routinely de waxed to water: xylene for 10 min (twice) → anhydrous ethanol for 2 min (twice) → 95% ethanol for 2 min → 80% ethanol for 2 min → 50% ethanol for 2 min → distilled water for 2 min. An appropriate amount of 50 μM/L MDC solution (diluted with DMSO) was dripped onto the paraffin section and incubated in an oven at 37 °C in a dark environment for 30 min, then washed with PBS solution for 3 times, 5 min each time. After air-drying the slides, the occurrence of autophagosomes was observed and photographed under fluorescence microscope (Olympus, Japan).

2.9. Fecal sample collection and 16S rRNA gene sequencing

Feces were collected from the rat colon, frozen in liquid nitrogen, and stored at −80 °C in a refrigerator. Total DNA from fecal microorganisms was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany). The quality of DNA extraction was assessed using 0.8% agarose gel electrophoresis, while DNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Fitchburg, WI). The DNA fragments were amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGAC-TACHVGGGTWTCTAAT-3′). PCR amplification, sequencing library preparation and high-throughput sequencing were performed by Personal Biotechnology, Co., Ltd (Shanghai, China).

2.10. Data analysis

16S rRNA raw sequencing data were processed and evaluated using the software QIIME (version: 1.8.0) (Caporaso et al., 2010). First, QIIME software is used to identify questionable sequences, then, USEARCH (version: 5.2.236) is called by QIIME software (version: 1.8.0) to check and reject chimeric sequences. Second, using QIIME software to invoke UCLUST, a sequence comparison tool, the previously obtained sequences were merged and OTUs were divided according to 97% sequence similarity, and OTUs with abundance values lower than 0.001% of the total sequenced amount of the whole sample were removed (Bokulich et al., 2013). Using the Mothur software, the statistical algorithm of Metastats was invoked to perform a two-comparison pairwise test of the differences in the amount of sequence between samples for each classification unit at the gate and genus level. A correlation matrix of microbiome composition with hormonal and autophagy indicators was created using Pearson’s correlation coefficient. A heat map of the correlation matrix was created using R software (version: 3.5.3). The interaction between F and As was analyzed by SPSS 22.0 software (IBM, Chicago, IL, USA).

2.11. Statistical analysis

The experimental data are expressed as mean ± standard deviation (X±SD). First, the normal distribution test and variance homogeneity test were performed on the data. When the test was in line with the normal distribution and variance homogeneity, the T test was used. Wilcoxon symbol rank test was used when the data did not conform to normal distribution and homogeneity of variance. One-way ANOVA and pairwise comparison were used for the comparison among groups. Data analysis was conducted in SPSS 22.0 software (IBM, Chicago, IL, USA), and P < 0.05 was considered statistically significant.

3. Results

3.1. Effects of F and/or As on testicular tissue and organ coefficients in rats

As shown in Fig. S1, compared with the control group, the organ coefficient of testicular tissue exposed to F group was significantly lower (P < 0.05). Compared with the control group, the testicular coefficient of the As group and the combined group also decreased, with no significant difference.

3.2. Histomorphological changes induced by F and/or As in progeny testicular tissue

Fig. 1 shows HE staining results of testicular tissue (40×). In the control group (a): Normal testicular tissue structure in the control group. The morphology of the spermatogenic tubules was regular, and the spermatogenic cells at all levels were clearly arranged and orderly. However, F exposure group (b): The damage of testicular tissue was obvious. The number of spermatozoa decreased significantly in the spermatogenic tubules, accompanied by vacuolization and shedding of immature germ cells. As exposure group (c): The boundary membrane of the germinal tubules was significantly thickened, and the number of spermatozoa in the germinal tubules was reduced. Immature germ cells were shed and germ cell arrangement was disordered. F+As exposure group (d): The testicular tissue was less severely damaged. A small amount of spermatogenic cell loss occurs and the number of spermatozoa in the lumen was reduced.

3.3. Effects of F and/or As on serum hormone levels in rats

As shown in Fig. 2, compared with the control group, F exposure group resulted in a significant decrease in serum FSH level (P < 0.05), while LH and T hormone levels were not significantly changed. In the As exposure group, the serum levels of FSH, LH and T hormone were significantly decreased (P < 0.05). In the group exposed to F+As, the serum T hormone level in rats was significantly decreased (P < 0.05), while FSH and LH levels were not significantly changed. In terms of serum testosterone levels, they were significantly lower in both the As and F+As groups compared to the F group (P < 0.05).

3.4. Relationship between microbiological composition and serum hormone levels in rats

So as to investigate the functional correlation between intestinal flora composition and testicular injury, we generated correlation matrices based on Pearson correlation coefficients between composition at the bacterial genus level and reproduction-related hormones. As shown in Fig. 3, based on the composition and sequence distribution of each sample, through Metastats analysis, we selected bacterial flora with significant difference at the generic level among each exposed group and analyzed its correlation with reproductive FSH, LH, and T hormones. The results showed that Corynebacterium was positively correlated (P < 0.05) with T hormones levels. LH was positively correlated with...
Fig. 1. HE staining results of testicular tissue of offspring rats (40×). Control group (a), F exposure group (b), As exposure group (c), and F and As co-exposure group (d). Spg: spermatogonia; Spec: spermatocytes; Sp: spermatozoa.

Fig. 2. Serum levels of FSH (a), LH (b) and T (c) in male rats of control group, F group, As group and F and As co-exposure group. Data were expressed as mean ± SD.

*Statistically significant differences compared to the control group (*P < 0.05).
detected by qPCR and western blotting, respectively (Fig. 4). Compared levels of autophagy-related genes like Beclin1, LC3 and p62 were

Interestingly, the mRNA expression level of p62 ($P < 0.01$) was significantly up-regulated exposed to F group ($P < 0.05$) and p62 ($P = 10.960$, $P < 0.05$) indicated an interaction between F and As (Fig. 7a). Likewise, the protein expression results of the autophagy-related genes Beclin1 ($F = 145.763$, $P < 0.05$), LC3 ($F = 63.127$, $P < 0.05$) and p62 ($F = 1.671$, $P = 0.21$) also provide evidence (Fig. 7b).

4. Discussion

F and As are common environmental contaminants that widely distributed all around the world. Studies have shown that high concentrations of F and As are present in groundwater in many areas. For example, the concentrations of F and As in groundwater in some parts of Mexico vary roughly between 0.24 and 1.8 mg/L and 0.12–0.65 mg/L, respectively (Sarinana-Ruiz et al., 2017). Similarly, groundwater in parts of Pakistan is contaminated with F (5.5–29.6 mg/L) and As (5.9–507 μg/L) (Rasool et al., 2015). The concentrations of F and As in the groundwater in the area exceeded the limit values set by the World Health Organization (50 μg/L for As and 1.5 mg/L for F). Furthermore, studies have shown that exposure to F and As alone can cause testicular tissue damage, declined testosterone levels, decreased sperm count, and reproductive damage in rats (Cao et al., 2016; Lima et al., 2018). In general, animals have a higher tolerance than humans. In this study, NaF (100 mg/L) and NaAsO$_2$ (50 mg/L) were chosen as exposure doses (approximately 25–50 times the human exposure dose) to establish an animal model, in order to investigate the effects of high concentrations of F and As on the reproductive system and to fill the gap in the study of testicular damage by F and As co-exposure. Our research group has successfully verified the model in the early stage (Tian et al., 2020).

In our study, we found that compared with the control group, F exposure alone resulted in significantly lower testicular organ coefficient in rats, but no significant change in the co-exposure group. An
intact testicular structure is fundamental for the normal development of the reproductive system. We observed by HE staining of histopathological sections that the number of spermatozoa in the testicular germinal tubules of rats exposed to F or As alone was significantly reduced, accompanied by germ cell abscission. This suggests that fluoride or arsenic exposure caused degenerative necrosis of germ cells and caused decreased testicular function, which is consistent with the findings of Zhang et al. (2016a, 2016b). However, the combined exposure caused less damage to testicular structures and weaker damage to germ cells than the individual exposures. This finding is similar to previous findings in the kidney (Tian et al., 2020). More notably, we found that exposure to F and As reduced serum levels of FSH, LH, and T hormones in rats, which may be achieved by regulating the hypothalamic-pituitary-gonadal axis through oxidative stress and endocrine disruption (Niu et al., 2015; Guvvala et al., 2016). Interestingly, co-exposure to F and As had a more subtle alteration of testosterone hormone levels in serum compared to F exposure alone. This may be due to the interaction between F and As, attenuating the toxic effects of F and As (Mondal and Chattopadhyay, 2020). However, there is a lack of literature support to clarify the interaction between F and As, and we need to go further into it.

Autophagy is an important biological process for eukaryotic cells to degrade macromolecular proteins, misfolded proteins and damaged organelles. The present study revealed that F exposure alone can increase the expression levels of Beclin1 and LC3, which is consistent with the findings of Zhang et al. (2017), Feng et al. (2019). F exposure causes a decrease in p62 expression levels, which may be due to the accumulation of autophagosomes as a result of heavy binding of p62 to ubiquitin cargoes such as misfolded proteins, damaged organelles, etc. during selective autophagy (Lamark et al., 2017; Wu et al., 2018). This demonstrates that F can induce selective autophagy. Especially, high concentrations of arsenic have been found to induce autophagy in testicular mesenchymal cells, whereas arsenic did not induce autophagy significantly in the present study, which may be related to the selected dose (Liang et al., 2020). Recent animal literature has shown a dose-response relationship between As levels in the brain and As levels in drinking water, indicating blood-brain barrier permeability of As to the central nervous system (Xi et al., 2010). Hence, we speculate that there may be a
dose-response relationship between arsenic concentration in testicular tissue and arsenic concentration in drinking water due to the presence of a blood-testis barrier in testicular tissue. Notably, co-exposure to F and As reduced the expression levels of LC3 and p62 and inhibited the development of autophagy compared with the exposed group alone. We speculate that this may be due to an antagonistic interaction between F and As. Previous study reported that F and As exposure alone is more effective than co-exposure in inducing renal autophagy, and that F has a greater effect on autophagy than As (Guo et al., 2020; Tian et al., 2020). This is consistent with our findings.

Numerous studies have shown that changes in the composition of the intestinal flora can cause a variety of diseases (Ma et al., 2019; Wu et al., 2019). In this study, F and As exposure affected the composition of the rat intestinal flora. We found that F and As exposure resulted in significant differences in the composition of Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria, which play an important role in maintaining the balance of the intestinal flora. Notably, we found the following two key points. (1) Intestinal Flora and Autophagy: A study reported that psychosocial stress may exacerbate inflammatory bowel disease by modulating intestinal flora and inflammation and altering intestinal autophagy (Wang et al., 2019). It has also been shown that impaired autophagy in intestinal epithelial cells leads to imbalances in host-microbe interactions and changes in the composition of the intestinal flora (Yang et al., 2018). Additionally, reversal of altered intestinal flora influences the occurrence of autophagy and oxidative stress in the brain (Xu et al., 2018). In our study, we found that altered intestinal flora composition can be involved in the regulation of testicular autophagy, with SMB53 and Anaerostipes genera being strongly correlated. These results all indicate a strong correlation between changes in the composition of the intestinal flora and the development of autophagy.

(2) Intestinal Flora and Hormones: At the genus level, we found a significant negative correlation between Parabacteroides and alterations in follicle-stimulating hormone. A previous report illustrated that the intestinal flora regulates the level of gastrointestinal hormone secretion and influences the development of gastric cancer (Zhou and Yang, 2019). Zhang et al. showed that Parabacteroides are increased in the gut of patients with polycystic ovary syndrome and that Probiotic Bifidobacterium lactis V9 modulates the levels of LH and FSH through the gut-brain axis (Zhang et al., 2019). Accordingly, it is reasonable to speculate that F and As exposure can affect testicular development and induce reproductive toxicity by modulating the gut microbiota to influence reproductive hormone secretion levels. In addition, Corynebacterium is associated with changes in testosterone hormones, which providing a strong basis for our speculations again. Notably, the alteration abundance of both SMB53 and Corynebacterium correlated with altered reproductive hormones and testicular autophagy, which suggesting that SMB53 and Corynebacterium may be important for F and As exposure-induced reproductive toxicity.

The interaction between F and As is a non-negligible factor in studying the process of F and As co-exposure on organismal injury. In the present study, we found an interaction between F and As in testicular reproductive toxicity by analyzing the expression levels of autophagy-related genes. Also, in combination with the above results, we speculate that there may be an antagonistic interaction between F and As in their reproductive toxic effects. Nevertheless, the type of interaction between F and As needs to be confirmed by further studies.

In summary, the results of the present study revealed that F and As exposure can cause altered testicular autophagic flux and imbalance of the intestinal microbiota in rats. Furthermore, we found that intestinal flora may be involved in the regulation of reproductive hormones and testicular autophagy (Fig. S2). Inevitably, our study also has some shortcomings. We only considered individual pups in our analysis of Fig. 5. Representative image of autophagosome in rat testis tissue. Control group (a), F exposure group (b), As exposure group (c), and F and As co-exposure group (d). The yellow arrow shows the autophagic vacuole. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 6. Correlations between intestinal flora composition at genus level and autophagy-related gene levels. The highlighted yellow box indicates a significant difference correlation ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Interaction between F and As on the mRNA and protein experiment of LC3, Beclin 1 and p62.
pups and not pups from the same litter, and we will consider this carefully in our subsequent studies.

**Declaration of Competing Interest**

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.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112506.

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