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Exposure to acrylamide inhibits uterine decidualization via suppression of cyclin D3/p21 and apoptosis in mice

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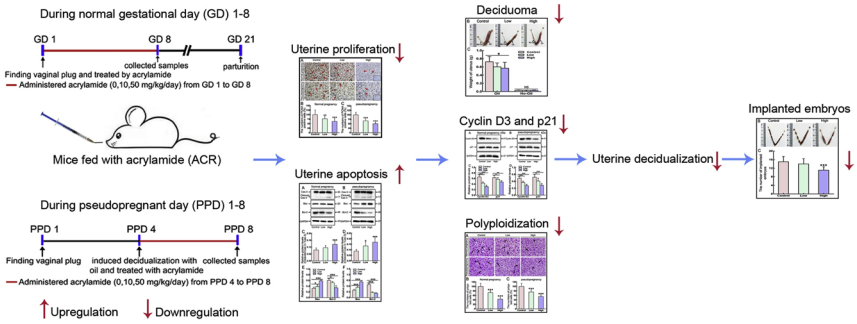
GRAPHICAL ABSTRACT



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ABSTRACT

Acrylamide (ACR), a neurotoxicity and carcinogenic chemical, has attracted considerable attention since it is present at high concentrations in thermally cooked carbohydrate-rich foods. ACR exposure significantly increased rate of fetal resorption, and decreased fetal body weights in mice. However, no detailed information is available about the effect of ACR on uterine decidualization, which is a vital process in the establishment of successful pregnancy. Thus, our aim of this study was to explore the effect and mechanism of ACR on uterine decidualization *in vivo* during mice pregnancy. Mice were gavaged with 0, 10, and 50 mg ACR /kg/day from gestational days (GD) 1 until GD 8, whereas pseudopregnant mice from pseudopregnant day (PPD) 4 until PPD 8. Results indicated ACR treatment dramatically reduced numbers of implanted embryos, and decreased the weights of implantation site and oil-induced uterus. Nevertheless, no significant difference was observed in the weights of no oil-induced uterus between control and ACR-treated group. Furthermore, ACR significantly reduced numbers of polyploidy and PCNA-positive decidual cells and expression of cyclin D3 and p21 proteins,

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and induced apoptosis of decidua, as presented by up-regulation of Bax and cleaved-caspase-3, and decreased Bcl-2 protein during normal pregnant and pseudopregnant process. In summary, ACR exposure significantly inhibited uterine endometrial decidualization *via* the apoptosis and suppression of cyclin D3/p21 in mice.

1. Introduction

Acrylamide (ACR) is a chemical used primarily in laboratories and various industrial processes, such as waste and drinking water treatment, paper manufacture, production of cosmetics and sugar (Exon, 2006). In 2002, Tareke et al. found that ACR is formed in high temperatures cooked carbohydrate-rich foods including potato chips, French fries, bread and other grain-based foods, through the Maillard Reaction between sugars and asparagines (Tareke et al., 2002; ALjahdali and Carbonero, 2019). Studies with laboratory-heated foods showed that the highest level of ACR was about 4.215 mg/kg in potatoes and beetroot (Tareke et al., 2002). In addition, tobacco smoke was demonstrated to be the second most source of ACR exposure after heated starchy food. The estimated average intake from cigarette smoking in adult smokers is 0.15-0.18 μg/kg/day ACR in Poland (Mojska et al., 2016). These findings have raised widespread concerns over the effect of ACR on human health. Apart from its neurotoxicity and carcinogenicity (Manjanatha et al., 2015; Hogervorst et al., 2017; Zhao et al., 2017; Komoike and Matsuoka, 2019), some studies have recently reported that dietary intake of ACR and fried potato chip (FPC) also increased the potential risk of reproductive and developmental toxicity (Wei et al., 2014; El-Sayyad et al., 2011).

Sprague-Dawley male rats and mice exposure to ACR led to reduced sperm numbers and viability and testosterone levels for 8 consecutive weeks and 35 days, respectively (Pourentezari et al., 2014; Wang et al., 2010). Wei et al. showed that female mice exposed to ACR led to the reduction of absolute and relative weights of uteri and ovaries and the numbers of corpora lutea and serum progesterone (Wei et al., 2014). Epidemiological investigations reported that ACR intake was statistically inversely associated with total and free estradiol levels and significantly positively associated with follicle-stimulating hormone level in premenopausal Japanese women (Nagata et al., 2015). In addition, increasing evidence has indicated that ACR had adverse effects on the development and maturation of oocyte. Mice fed with ACR-containing diet for 6 weeks caused aberrant oocyte cytoskeletons and reduced germinal vesicle breakdown and polar body extrusion rates (Duan et al., 2015). Maturation of oocytes was markedly impaired in ACR-treated female mice for 7 days or oocytes exposed to different doses of ACR *in vitro* (Liu et al., 2015; Aras et al., 2017). Furthermore, dams treated with ACR or FPC significantly increased the rate of abortion and neonatal mortality, and reduced litter size and body weight of fetuses (El-Sayyad et al., 2011; Duan et al., 2015; Yu et al., 2019).

In humans and rodent animals, fetal exposure to ACR through the diet may start in uterus, because ACR has been found to cross placental barrier *in vitro* as well as *in vivo* (Duarte-Salles et al., 2013; Sorgel et al., 2002; Schettgen et al., 2004; von Stedingk et al., 2011). What's more, cohort studies showed that maternal dietary ACR intake was negatively related to fetal growth such as reduced birth weight and head circumference and small for gestational age (SGA) (Duarte-Salles et al., 2013; Kadawathagedara et al., 2016; Pedersen et al., 2012). When mice blastocyst penetrates the uterine epithelium into the stroma on GD 5, it can induce uterine decidualization by stromal cell proliferation, and differentiation into polyploidy decidual cells, which is a vital process in the establishment of successful pregnancy during embryo implantation (Tan et al., 2002). Cyclin D3, a G1 phase cell-cycle regulatory protein, is involved in the proliferation and decidualization of stromal cells (Tan et al., 2002; Sroga et al., 2012a; Li et al., 2008). Coordinated interaction of cyclin D3 with p21 (a cell differentiation factor), is crucial to the development of uterine stromal cell decidualization and polyploidy (Tan et al., 2002; Li et al., 2008). Surprisingly, little is known about the effect of ACR on the uterine decidualization. Therefore, our aim of this study was to investigate the effect and mechanism of ACR on the uterine decidualization *in vivo* during mice pregnancy.

During decidualization process, cell proliferation and apoptosis occur simultaneously in uterine tissues, and the relative extent of both processes is fundamental to normal pregnancy (Correia-da-Silva et al., 2004). Increased levels of apoptosis have been reported to be associated with miscarriage (Boeddeker and Hess, 2015; Kokawa et al., 1998), SGA (Barrio et al., 2009) and intrauterine growth restriction (Kokawa et al., 1998). Apoptosis is regulated by various proteins, such as the Bcl-2 protein family, which consists of both anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax expressed in the deciduas of rodent uterus during early gestation (Theron et al., 2013). In addition, the activation of caspase-3, a critical executioner, is known to play a crucial role in apoptosis (Correia-da-Silva et al., 2004; Boeddeker and Hess, 2015). To further explore the role of apoptosis in the uterine decidualization of mice treated by ACR, the expression levels of cleaved caspase-3, Bax and Bcl-2 were assessed by western blotting.

2. Materials and methods

2.1. Chemicals and reagents

Sesame oil (S3547) and ACR (A8887), a white and monomer dry crystal, were purchased from Sigma-Aldrich (St. Louis, USA). Rabbit anti-Bcl-2 was obtained from Zsbio (Beijing, China). Rabbit anti-Caspase3, Bax, Cyclin D3, PCNA, and GAPDH were purchased from Cell Signaling Technology (Boston, USA). Mouse p21 (B-2: sc-271532) was purchased from Santa Cruz Biotechnology (Texas, USA). Radioimmunoprecipitation assay (RIPA) and a phosphatase inhibitor cocktail were purchased from PPLYGEN (Beijing, China). PMSF was from Solarbio (Beijing, China).

2.2. Animals and administration of ACR

The study was approved by the Animal Ethics Committee of Nanchang University. All mice were treated humanely according to the guidelines for laboratory animal science at Nanchang University. Mature mice (25 − 30 g, Kunming strain) were obtained from the Laboratory Animal Center of Jiangxi traditional Medical University. Mice were maintained at room temperature with 12-h dark-light cycles and free access to food and water. After 3 days of acclimatization, female mice were mated with fertile males or vasectomized male mice of the same strain to induce normal pregnancy or pseudopregnancy, respectively (day of finding vaginal plug = day 1). Normal pregnant mice were divided randomly into three groups (n = 10/group) and treated with ACR dissolved in distilled water (0, 10 and 50 mg/kg/day) daily by oral gavage at 7:00 AM from gestational day (GD) 1 to GD 8 (Fig. 1A) (Duan et al., 2015; Yu et al., 2019). These doses were selected because estimations of the Food and Agriculture Organization/World Health Organization (WHO) panel of the average intake of ACR for the general public ranged between 0.3 to 0.8 mg/kg-bw per day through food intake, and the exposure level of children and adolescents to ACR is 2–3 times higher than adult consumers in the US (Programme, 2002) and the US Food and Drug Administration issued preliminary exposure estimates of food-derived ACR, which were 0.43 and 1.06 mg/kg-bw per day for adults and children, respectively (Doerge et al., 2008). Additionally, exposure *via* occupation and environment can also increase this exposure. Furthermore, human dose converts to a mice equivalent dose based on the body surface area (Reagan-Shaw et al., 2008) and

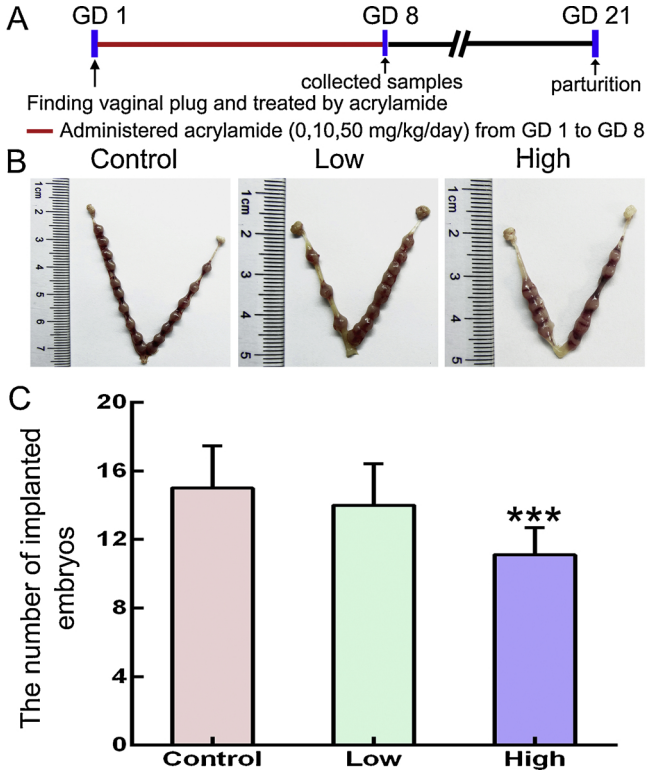

Fig. 1. ACR exposure reduces the numbers of implanted embryo.
(A) Diagrammatic representation of ACR treatment protocol. Female mice were mated with fertile male to induce pregnancy (day of finding vaginal plug = gestational day 1, GD 1). Pregnant dams were gavaged with ACR from GD 1 to GD 8. Finally, the samples were collected on GD 8. (B) Representative images of uteri from pregnant mice exposed to 0 (control), 10 (low-dose), and 50 (high-dose) mg/kg/day ACR from gestational day (GD) 1 until GD 8. (C) The numbers of implanted embryos on GD 8. Results are shown as mean ± SD (n = 10 mice/treatment group). *** P < 0.001 compared with control group.

internal exposure from endogenous ACR formation can further increase this dose closer to 10 mg/kg/day. The basis for selecting the highest concentration is that 50 mg/kg/day is a maximally tolerated dose producing hind limb paralysis (Camacho et al., 2012). The control group mice were given the same volume of distilled water. Mice were anesthetized with pentobarbital sodium prior to cervical dislocation and uterine collection between 16:00 and 17:00 on GD 8.

Additionally, pseudopregnant mice were induced the artificial decidualization by intraluminally infusing 25 µl of sesame oil into one uterine horn on pseudopregnancy day (PPD) 4, whereas the contralateral uninjected horn served as a control (Gao et al., 2007). Simultaneously, induced decidualization mice were also assigned randomly into three groups (n = 10/group) and administered with ACR dissolved in distilled water (0, 10 and 50 mg/kg/day) daily by oral gavage from PPD 4 to PPD 8 (Fig. 3A). Finally, experimental mice were euthanized prior to cervical dislocation, and the uteri were collected on PPD 8 and photographed and weighed and stored at -80 ℃ until further analysis.

2.3. H&E staining and polyploid analysis of decidual cells

The uteri of implantation site and artificially induced decidualization were fixed in Bouin's solution overnight, dehydrated in gradient alcohol, cleared in xylene, embedded in paraffin and sectioned serially at 5 µm. Sections were mounted and stained with H&E for morphological observation and polyploid analysis of decidual cells using a Nikon DS-Fi1 microscope (Nikon, Japan). The decidual cell polyploidy is characterized by the formation of large mono or bi-nucleated cells, a characteristic of nuclear endoreduplication (Tan et al., 2002). The numbers of polyploid decidual cells were calculated in five non-overlapping fields at magnification, x400 by Image J software (v. 1.48, National Institutes of Health, Bethesda, MD). The procedure was repeated in 3 specimens from each mouse. The mean number of cells was calculated per mouse, then per treated group.

2.4. Immunohistochemical staining

Tissue sections were deparaffinized in xylene, and hydrated in

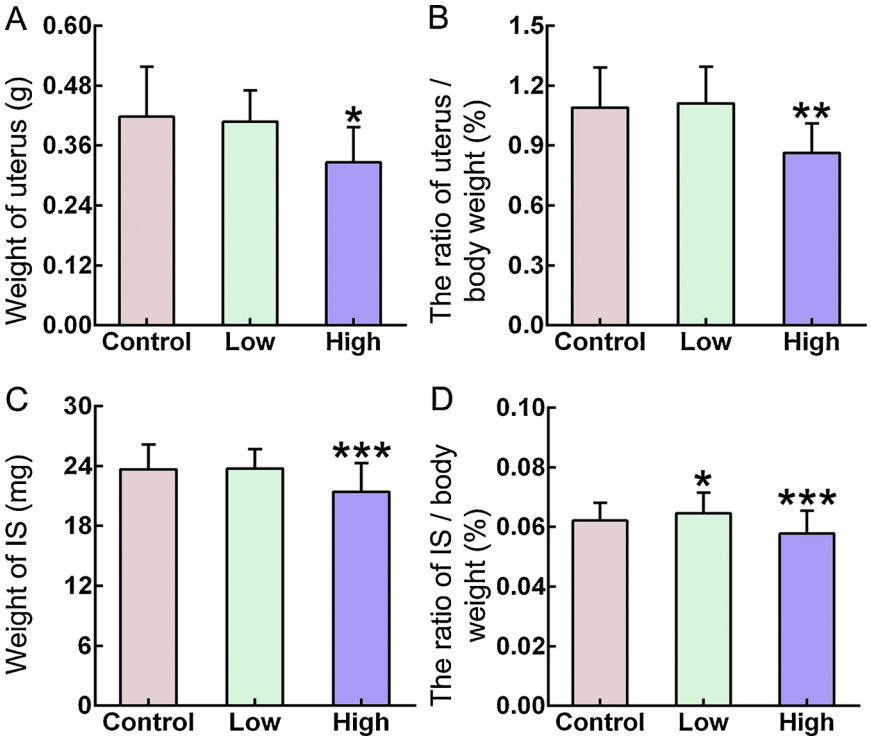

Fig. 2. The effect of ACR on the weight of uterus and implantation site.
(A) The weight of uterus. (B) The ratio of uterus weight *vs.* the body weight. (C) The weight of implantation sites (IS). (D) The ratio of IS weight to body weight. Results are shown as mean ± SD (n = 10 mice/treatment group). *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group.

graded ethanol solutions. Then, sections were bathed in EDTA solutions and microwaved for antigen retrieval. Endogenous peroxidase was inactivated by incubating in 3 % hydrogen peroxide for 10 min. Nonspecific reaction was blocked in 5 % BSA for 50 min. Sections were incubated with rabbit anti-PCNA antibody (1:500) overnight at 4 ℃, followed by incubation in horseradish peroxidase-conjugated secondary antibody (1:150) for 45 min at 37 ℃. Positive signals were detected with fresh DAB solution (ZSGB-BIO, China). In some slices, the primary antibodies were replaced with rabbit preimmune IgG as a negative control. Experimental results were observed by light microscopy and quantification of PCNA-positive cells was calculated by Image J software (v. 1.48, National Institutes of Health, Bethesda, MD). Each slide was measured in randomly selected five fields under the same magnification (×400). 3 samples /mice were used, and 5 serial sections were used to calculate the mean positive cells of per samples. Mean positive cells for per group were calculated from 6 mice.

2.5. Western blotting

Uterine tissues were homogenized and lysed in the ice-cold RIPA lysis buffer supplemented with PMSF solution, then centrifuged at 12,000 rpm for 10 min at 4 ℃. Protein in the supernatants were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes by electrophoresis. The membranes were blocked with 5 % skim milk in Tris-buffered saline Tween (TBST) for 1 h to block nonspecific bindings and incubated overnight at 4 ℃ with the primary antibodies: GAPDH (1:8000), Bax (1:1000), Bcl-2 (1:1000), Caspase-3 (1:500), and p21 (1:5000). After washing in TBST 10 min for 3 times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) at 37 ℃ for 45 min and visualized *via* enhanced chemiluminescence (Pierce, USA). Signal quantification were determined by the densitometry of protein bands using Quantity One software and normalized to GAPDH.

2.6. Statistical analysis

All data were analyzed by using GraphPad Prime 6 or SPSS version 21.0 (SPSS Inc, Chicago, IL, USA) and presented as means ± standard deviations (SD). Normal distribution of data was checked by ShapiroWilk test. The statistical significance of normally distributed data was determined by using one-way analysis of variance (ANOVA) followed by LSD's post-hoc test. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. ACR exposure reduces the numbers of implanted embryo

Compared to control group, low-dose ACR exposure had no significant effect on the numbers of implanted embryo. However, high-dose ACR treatment dramatically reduced the numbers of implanted embryos on GD 8 (Fig. 1B and C).

3.2. The effect of ACR on the weights of uterus and implantation site

There were no significant difference in absolute and relative uterine weights between the control and low-dose group (Fig. 2A and B). However, high-dose ACR treatment markedly decreased the absolute and relative uterine weights (Fig. 2A and B). To further investigate the effect of ACR on the uterine decidualization, we also examined the effect of ACR exposure on the weights of implantation site and artificially induced decidualizing uteri. As shown in Fig. 2C and D, high-dose ACR treatment significantly reduced absolute and relative uterine weights of implantation site. Furthermore, high-dose ACR also prominently attenuated the weight of oil-induced uterus (Fig. 3B and C). Nevertheless, no significant difference was observed in the weights of no oil-induced uterus between control group and ACR-treated group (Fig. 3B and C).

3.3. ACR exposure reduces the numbers of polyploidy and proliferative decidual cells

H&E staining showed that the numbers of polyploidy decidual cells were significantly lower in the ACR-treated groups than those in the control group during normal pregnant process (Fig. 4A and B). Furthermore, during pseudopregnant process, ACR exposure also reduced dramatically the numbers of polyploid decidua cells induced by oil in both low and high-dose groups (Fig. 4A and C). However, in no-oil induced uteri, there was no obvious difference in the numbers of polyploidy cells between control and ACR-treated group (Supplemental Fig. 1). In addition, *in vitro* analysis of stromal cells by the flow cytometer after DNA staining also revealed that ACR treatment remarkably reduced the proportion of multinucleated cells (4C and > 4C) compared to control group (Supplemental Fig. 2 A and B), which is consistent with the results of *in vivo* experiments.

Next, we examined the effect of ACR treatment on the proliferation of decidual cells. Compared to control group, the numbers of PCNA-positive cells were remarkably decreased in the ACR-treated group during the normal pregnant (Fig. 5A and B) and pseudopregnant process (Fig. 5A and C).

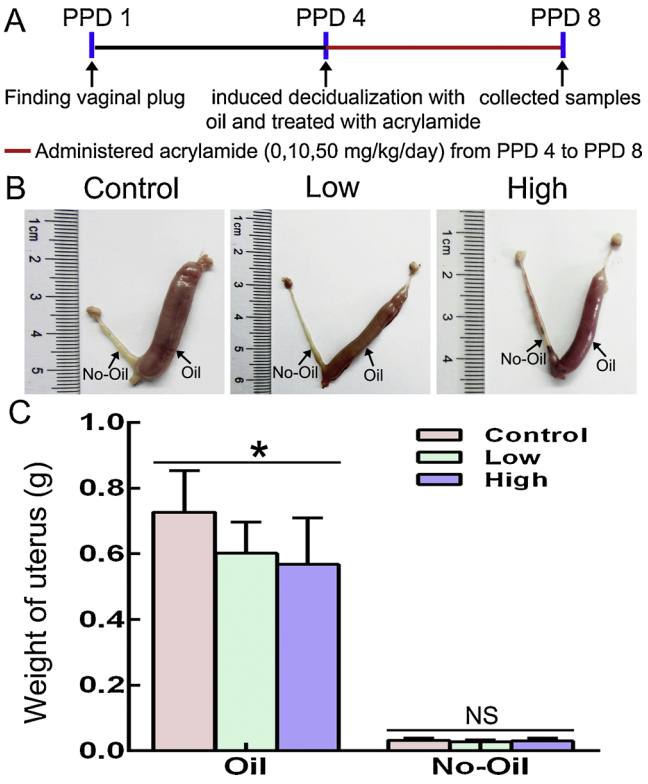

Fig. 3. The effect of ACR on the weight of no oil and oil-induced uterus. (A) Experimental design of artificially induced decidualization and ACR exposure protocol. Adult females were mated with vasectomized male to induce pseudopregnancy (day of vaginal plug = pseudopregnancy day 1, PPD 1). Pseudopregnant mice were induced the artificial decidualization by intraluminally infusing sesame oil into one uterine horn on PPD 4 and simultaneously gavaged with ACR from PPD 4 to PPD 8. Finally, the samples were collected on PPD 8. (B) Representative images of uteri from no oil and oil-induced decidualization mice exposed to 0 (control), 10 (low-dose), and 50 (high-dose) mg/kg/day ACR from PPD 4 until PPD 8. (C) The weight of no oil and oil-induced uterus. Results are shown as mean ± SD (n = 10 mice/treatment group). *$P < 0.05$ compared with control group. NS: no statistical significance.

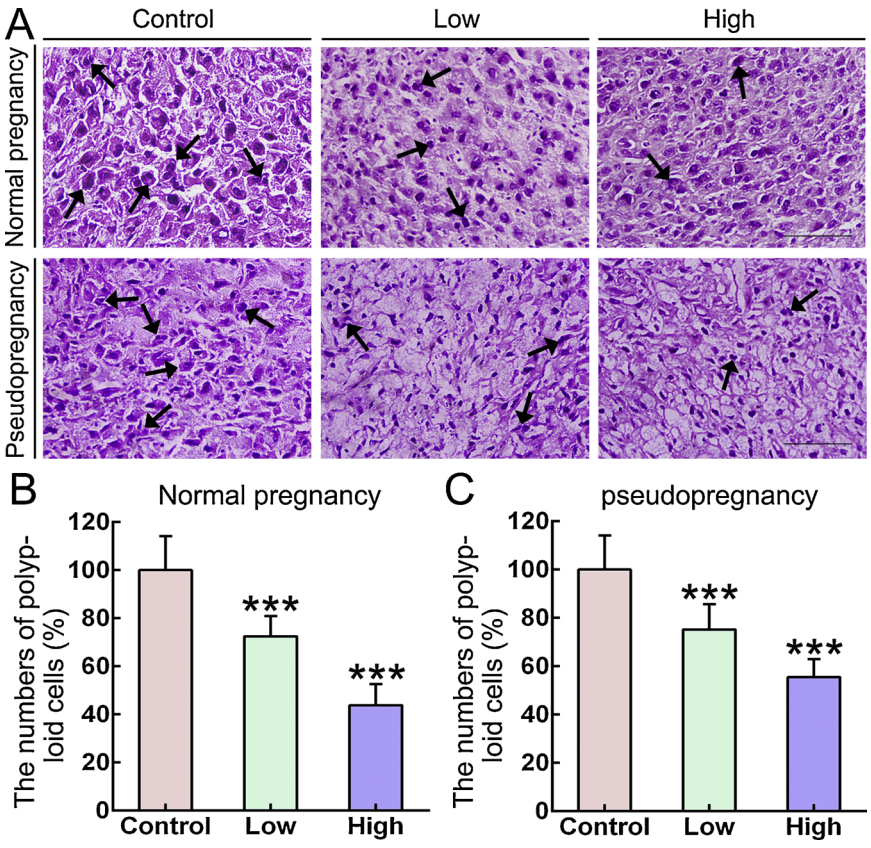

Fig. 4. Exposure of ACR reduces the number of polyploid decidual cells.
(A) Representative images of H&E-staining uterine endometrium from mice exposed to 0 (control), 10 (low-dose), and 50 (high-dose) mg/kg/day ACR during normal pregnant and pseudopregnant process. Black arrows: polyploid decidual cells. Scale bar, 50 μm. (B, C) The numbers of polyploid decidual cells on gestational day 8 (B) and pseudopregnancy day 8 (C). Results are shown as mean ± SD (n = 18 specimens/6 mice/treatment group). ***P < 0.001 compared with control group.

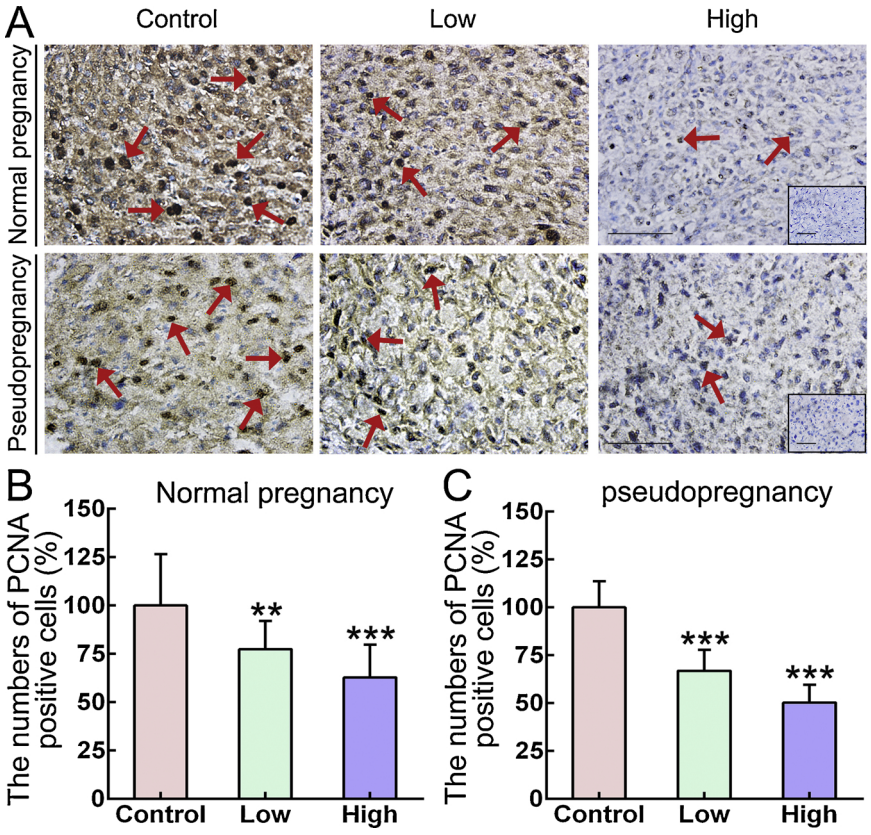

Fig. 5. The effect of ACR on the numbers of PCNA-positive cells.
(A) Representative images of PCNA immunohistochemical staining uterine sections from mice exposed to 0 (control), 10 (low-dose), and 50 (high-dose) mg/kg/day ACR during normal pregnant and pseudopregnant process. Photos inserted in high-dose group served as negative controls. Red arrows: PCNA-positive cells. Scale bar, 50 μm. (B, C) The numbers of PCNA-positive cells on normal gestational day 8 (B) and pseudopregnancy day 8 (C). Results are shown as mean ± SD (n = 18 specimens/6 mice/treatment group). **P < 0.01 and ***P < 0.001 compared with control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. ACR exposure down-regulated expression level of cyclin D3 and p21 proteins

Western blot analysis showed that expression levels of cyclin D3 and p21 proteins were significantly down-regulated in the ACR-treated groups compared with control group during the normal pregnant (Fig. 6A and C) and pseudopregnant processes (Fig. 6B and D).

3.5. ACR exposure induced the apoptosis of uterus during normal and pseudopregnant processes

Western blot results indicated caspase-3 was cleaved to active fragments cleaved-caspase-3 after ACR exposure, and expression levels of cleaved-caspase-3 protein were significantly up-regulated in a dose-dependent manner during the normal pregnant (Fig. 7A and C) and pseudopregnant processes (Fig. 7B and D). Moreover, apoptotic protein Bax was also increased obviously, but anti-apoptotic protein Bcl-2 dramatically decreased in the ACR-treated groups compared with control group during the normal (Fig. 7A and E) and pseudopregnant processes (Fig. 7B and D). Additionally, TUNEL staining also showed low and high-dose ACR significantly increased apoptotic positive cell numbers of decidual tissues during the normal pregnancy (Supplemental Fig. 3 A and B) and pseudopregnancy (Supplemental Fig. 3 A and C).

4. Discussion

Recently, evidence on the adverse effect of ACR or FPCs on the pregnancy including an augment in the resorbed rate and neonatal mortality and SGA, and a reduction in birth weight and prenatal and postnatal survival of fetuses is mounting. However, whether it is associated with alteration of uterine decidualization remains unknown. In this study, we firstly investigated the effect ACR on the mice uterine decidualization *in vivo* by assessing the numbers of implanted embryo, the weights of uterus and implantation site and oil-induced uterus, the numbers of polyploidy and PCNA-positive decidual cells, decidualization markers and uterine apoptosis. Our results revealed that feeding mice an ACR contaminated diet significantly inhibited uterine endometrial decidualization *via* the apoptosis and suppression of cyclin D3/p21 in mice.

ACR was delineated on the catalog in 1990 as a carcinogen and in 2011 as a reproductive toxicant (Becalski et al., 2003; Larsson et al., 2009). No significant risk level (NSRL) for ACR as a carcinogen is 0.21 μg/d in mice, but there is no NSRL for ACR as a reproductive toxicant (Becalski et al., 2003). Currently, our results showed that 10 mg/kg/day ACR exposure from GD1 to GD 8 did not significantly affect the numbers of implanted embryos, but a significant decrease of implanted embryos was observed under 50 mg/kg/day ACR treatment. When Fischer 344 female rats were exposed to ACR in drinking water at 0, 0.5, 2.0 and 5.0 mg/kg/day for 10 weeks until lactation, implantations and live pups/litter at birth were significantly reduced at 5.0 mg/kg/day (Tyla et al., 2000). This is almost consistent with Ferguson et al's result, in which resorbed phenomena were more common in Fischer 344 pregnant rats treated with 5 mg/kg/day ACR from GD 6 until parturition (Ferguson et al., 2010). Nevertheless, El-Sayyad et al. showed the rates of abortion in mice treated with 25 μg/kg/d ACR or a diet containing 33 % FPC from GD6 to parturition rose to 76.2 %, and neonatal mortality was observed in the treated groups but was higher in the FPC-treated group (El-Sayyad et al., 2011). These results suggested that effects of ACR on the pregnancy may be related to multiple factors such as exposure time, exposure dose and animal species.

Uterine decidualization, marked by stromal cell proliferation and differentiation into polyploidy decidual cells, is an essential prerequisite to support embryo growth and maintain early pregnancy (Tan et al., 2002; Paria et al., 2002). The weights of whole uterus and implantation site are a sign of uterine decidualization. Our results found that 50 mg/kg/day ACR treatment obviously decreased the absolute and relative weights of uteri and weights of uteri in the implantation site. This is similar to Wei et al. report, in which females treated with 20 and 40 mg/kg/day ACR 30 consecutive days showed a significant decrease in body weights and uterine weights (Wei et al., 2014). To further determine the effects of ACR on the decidualization, we utilized the model of artificial decidualization, in which it was induced by intraluminal injections of sesame oil in one uterine horn. Our results also evidenced that high-dose ACR obviously reduced the weight of oil-induced uterus. Nevertheless, there was no significant difference in the

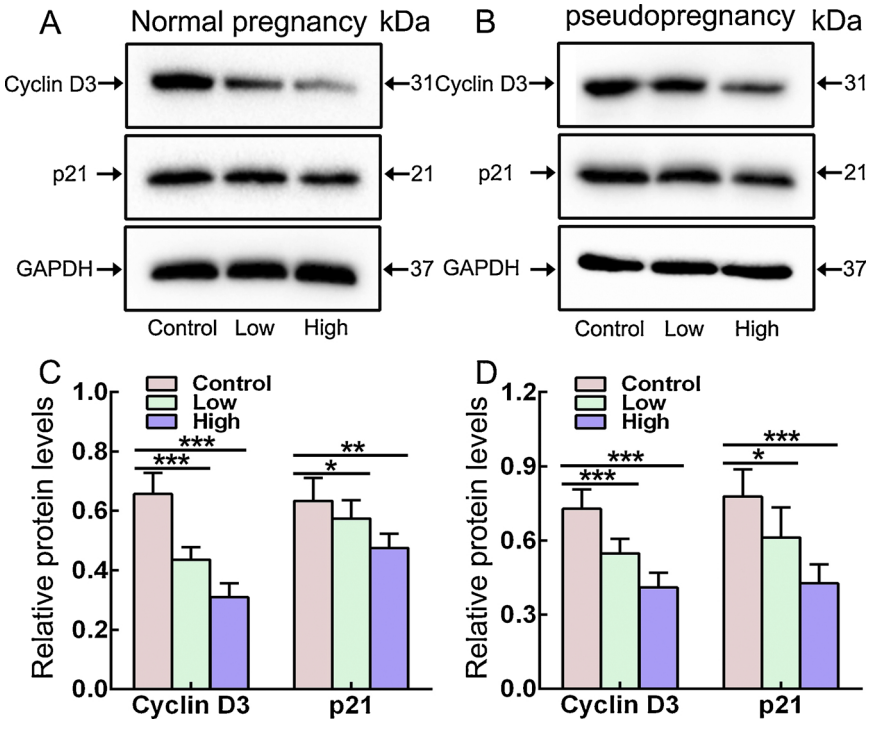

Fig. 6. The effect of ACR exposure on the expression levels of cyclin D3 and p21 proteins.
(A, B) Representative immunoblotting images of cyclin D3 and p21 protein during normal pregnant (A) and pseudopregnant process (B). (C, D) The relative protein levels of cyclin D3 and p21 during normal pregnant (C) and pseudopregnant process (D). Results are shown as mean ± SD (n = 6 uteri/6 mice/treatment group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control group.

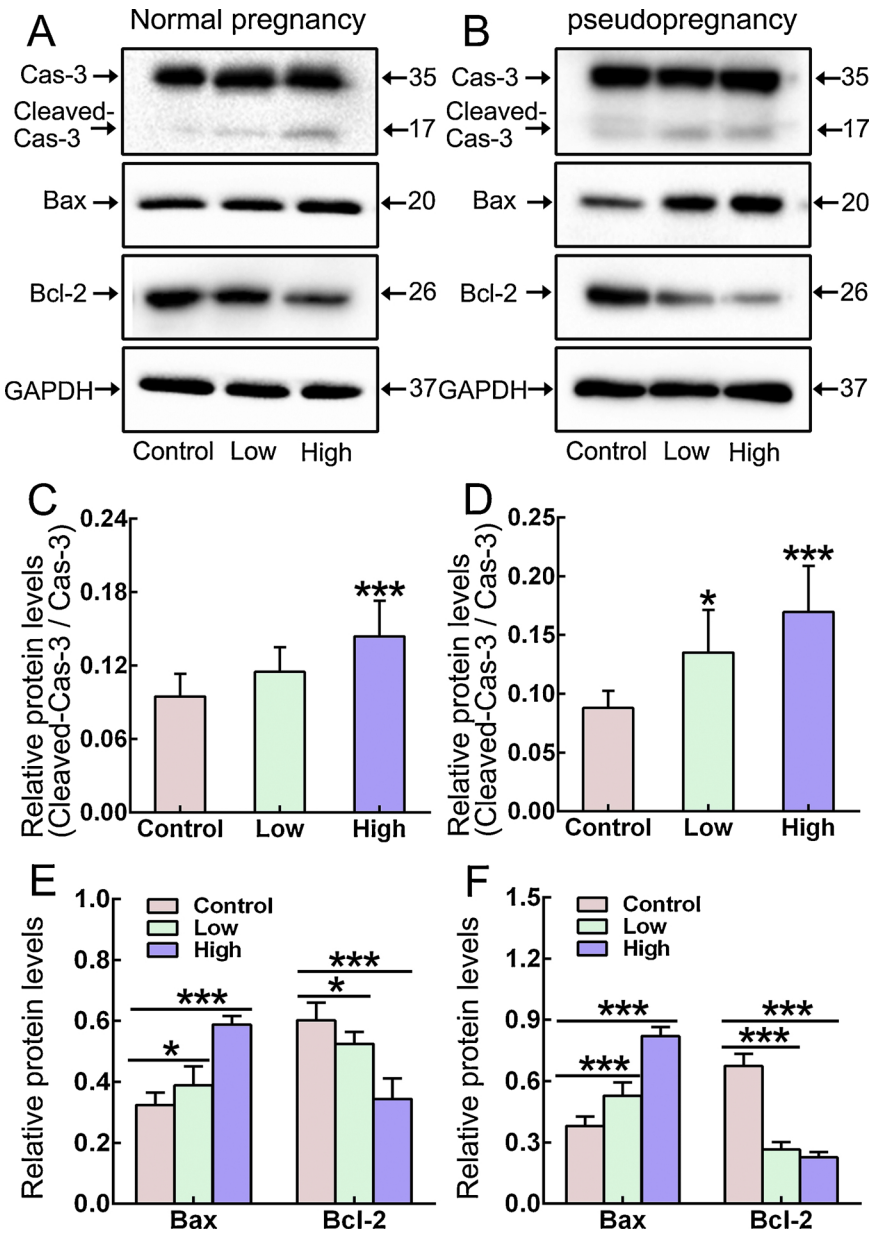

Fig. 7. ACR exposure induced the apoptosis of uterus during normal and pseudopregnant processes.
(A, B) Representative immunoblotting images of Caspase-3, Bax, Bcl-2 during normal pregnant (A) and pseudopregnant process (B). (C, D) The relative protein levels of cleaved-caspase-3 to Caspase-3 during normal pregnant (C) and pseudopregnant process (D). (E, F) The relative protein levels of Bax and Bcl-2 during normal pregnant (E) and pseudopregnant process (F). Results are shown as mean ± SD (n = 6 uteri/6 mice/treatment group). *$P < 0.05$, ***$P < 0.001$ compared with control group.

weights of no oil-induced uterus between the control and ACR-treated group. These results suggest that ACR is more harmful to undergoing decidualization uterus than non-decidualized endometrium, which may result in adverse gestational outcomes.

To further explore the mechanism of ACR on the decidualization, we detected the expression levels of cyclin D3 and p21 proteins, which are associated with proliferation and differentiation of decidual cells, respectively (Tan et al., 2002; Li et al., 2008). Moreover, complex of cyclin D3/ cdk6/p21 is involved in polyploidization of uterine stromal cells (Tan et al., 2002). Our immunoblotting results showed that 10 and 50 mg/kg/day ACR treatment all significantly decreased the levels of cyclin D3 and p21 proteins during the normal pregnant and pseudopregnant processes, which probably led to the down-regulation of the numbers of PCNA-positive cells and polyploidy decidual cells and the deficient of uterine decidualization in ACR-treated mice. Defective decidualization was also observed in interleukin11 receptor alpha null mice, which is also accompanied by a dramatic decrease of cyclin D3 and p21 proteins (Li et al., 2008). In line with the above finding, chen et al. found that treatment with ACR for 48 h resulted in a decrease of cyclin D1 and p21 proteins and proliferation inhibition and cell-cycle arrest of human astrocytoma U-1240 MG cells (Chen et al., 2010). In addition, the expression levels of p21 were also significantly decreased in 50 mg/kg/day ACR-treated male mouse live (Chen et al., 2018). However, 100 μM ACR treatment significantly increased expression of cyclin D1 and D3 and the proliferation of HepG2 cells (Shan et al., 2014). These results suggest that high concentration ACR may inhibit cell proliferation, whereas low concentration ACR may be beneficial to promoting cell growth and will lead to tumorigenesis as a carcinogen. Nevertheless, the potential mechanism of its action remains to be further explored.

Decidualization begins at the time of blastocyst attachment in mice on GD 4.5 (Sroga et al., 2012b). During the next 3 days of pregnancy, decidual cells surrounding the site of implantation proliferate and differentiate extensively, finally developing into larger, often with bi-nucleated or polyploid status (Tan et al., 2002; Ramathal et al., 2010). The causes of defective decidualization exposure to ACR may also be related to the proliferation and apoptosis changes of uteri. Our results demonstrated that ACR treatment significantly inhibited the proliferation of uterine stromal cells and reduced the numbers of polyploidy and induced apoptosis of decidua, as shown by increased the expression of Bax and cleaved-caspase-3, and decreased Bcl-2 protein during normal pregnant and pseudopregnant process. Furthermore, our previous study showed that gestational exposure to ACR inhibited proliferation and induced apoptosis of placentas on GD 13, as shown by decreased Ki67-positive cells and Bcl-2 protein, and increased the expression of Bax, cleaved-caspase-3, and cleaved-caspase-8 proteins (Yu et al., 2019). Kacar et al. also found that 4.6 mM ACR decreased cell viability and induced apoptosis in 64 % of the A549 cells *in vitro* (Kacar et al., 2018). In addition, Sellier et al. reported that *in vitro* chronic exposure to 10 u M ACR over 3 months accelerated human endothelial senescence (Sellier et al., 2015). However, Huang et al. showed that ACR could reduce cardiomyocyte proliferation rather than cell apoptosis (Huang et al., 2018). On the basis of these studies, we believe that inhibition of proliferation and apoptosis induction may be one of the causes of decidualization disorder in ACR-treated mice. However, underlying mechanisms of ACR-induced decidual apoptosis in pregnant mice requires further research.

At present, our results are only limited to the effect of ACR on uterine decidualization in mice. These results will require further confirmation about the function and mechanism of ACR on the decidualization of human endometrium because there are some differences in the decidual process between mice and human (Ramathal et al., 2010). In contrast to rodent animal, decidualization of human endometrium does not require external stimulation such as embryo implantation, sesame oil and glass beads. Instead, this process is triggered by the upregulation of progesterone and local cAMP production during secretory phase of menstrual cycle (Ramathal et al., 2010; Gellersen and Brosens, 2014). Furthermore, future studies should be conducted over whether ACR exposure through diet was associated with menstrual disorder and aberrant decidualization in large populations. In addition, the toxicological effect of ACR on endocrine function of corpus luteum should also be concerned. The steroid hormones progesterone and estradiol secreted by luteal cells play a pivotal role in directing the differentiation of stromal cells (Gellersen and Brosens, 2014), which may result in the alteration of uterine decidualization.

In summary, our findings evidence that gestational exposure to ACR significantly inhibits uterine endometrial decidualization *via* suppression of cyclin D3/p21 and proliferation and apoptosis induction in pregnant mice, which may result in adverse gestational outcomes. Therefore, pregnant females should reduce their exposure to environmental, occupational, dietary, and lifestyle sources of ACR.

CRediT authorship contribution statement

Dainan Yu: Conceptualization, Investigation, Methodology, Writing - original draft. **Qingyun Liu:** Investigation, Methodology, Writing - original draft. **Bo Qiao:** Investigation, Methodology, Writing - original draft. **Wenyu Jiang:** Data curation, Validation. **Lixia Zhang:** Investigation, Validation. **Xin Shen:** Investigation, Validation. **Liping Xie:** Data curation, Validation. **Hui Liu:** Data curation, Validation. **Dalei Zhang:** Resources, Supervision. **Bei Yang:** Conceptualization, Supervision, Writing - review & editing. **Haibin Kuang:** Conceptualization, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declared that there are no conflicts of interests of this work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121785.

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