Indole-3-methanol alleviates cisplatin-induced ovarian damage by inhibiting ovarian fibrosis through the TGF-β1/Smad pathway

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Research Article

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Abstract

Background Ovarian injury is one of the side effects of chemotherapy in female patients, which seriously endangers female reproductive health. Indole-3-carbinol (I3C), a natural substance abundant in cruciferous vegetables, has been reported to attenuate tissue damage. This study aimed to investigate whether I3C could prevent from ovarian damage induced by chemotherapy.

Methods A mouse model of ovarian damage was established by intraperitoneal injection of cisplatin or co-treatment with I3C. Then the ovarian index and estrous cycle was assessed. Meanwhile, follicles counting was conducted to evaluate the effect of I3C in follicular development. Also, we performed the TUNEL and IHC staining to analyze the level of apoptosis and fibrosis, respectively. Western blot and qRT-PCR was used as quantitative methods to evaluate the expression of relative markers and TGF-β1/Smad pathway. Hela cells and Caski cells was used to investigate the anti-tumor activity of I3C by cell counting kit-8, the wound healing assay and colony formation assay in vitro.

Results Our results showed that administration of I3C restored the ovary index and improved estrous cycle disorders. Follicle counting results showed that I3C is able to inhibit primordial follicles over-activation caused by cisplatin treatment, and maintained primordial follicle pool. We also found that I3C can down-regulate the levels of Bax and γH2ax, and inhibit the apoptosis of ovarian granulosa cells. In addition, I3C also reduced ovarian fibrosis and inhibited α-SMA and Collagen I expression levels. Further research revealed that I3C treatment significantly down-regulated the activity of the TGF-β1/Smad signaling pathway. Finally, we demonstrated that I3C could inhibit the proliferation, migration and colony formation of cervical cancer cells in vitro.

Conclusions In summary, I3C alleviates primordial follicular over-activation, granulosa cell apoptosis and ovarian fibrosis induced by cisplatin, and exhibits antitumor activity. Our study provides an innovative therapeutic strategy for preventing ovarian function from chemotherapy in female cancer patients.

Background

As the incidence of tumors rises year after year, so does the number of young oncology patients. At the same time, advances in cancer detection and treatment methods have improved cancer patients' prognoses and increased overall and disease-free survival rates (Bedoschi et al., 2019; Vo and Kawamura, 2021). However, there is growing evidence that anti-cancer treatment is frequently the cause of ovarian insufficiency and infertility (Bedoschi et al., 2019; Overbeek et al., 2017; Spears et al., 2019). Of particular concern to young women diagnosed with cancer is their reproductive potential after chemotherapy (Bedoschi et al., 2019). Improving oncology patients' reproductive health and preserving their fertility has become a difficult issue in the treatment process.

The length of a woman's reproductive life was determined by the amount of the primordial follicle (Findlay et al., 2015; Pepling, 2006). In contrast, chemotherapy-induced ovarian function impairment is primarily manifested by over-activation of primordial follicles, resulting in a decrease in ovarian reserve (Jang et al.,
Multiple variables affect the ovaries at the same time, reducing primordial follicular reserve, resulting in premature ovarian failure (POF) and fibrosis, which causes inflammation and further damage (Spears et al., 2019; Zhang and Liu, 2015). Meirow et al.'s histological studies of the human ovary revealed that localised ovarian cortical fibrosis and vascular injury may be additional significant mechanisms of chemotherapy-induced ovarian functional impairment (Meirow et al., 2007), but this hasn't been widely discussed yet. Additionally, Cui et al. showed that primary ovarian insufficiency causes ovarian tissue fibrosis, which is a pathogenic alteration (Cui et al., 2020). This might help in the creation of a fresh plan to stop chemotherapy-related ovarian harm.

Previous research has demonstrated that the TGF–β1/Smad signaling pathway is crucial for the development of hepatic, renal, cardiac, and pulmonary fibrosis (Chen et al., 2018; Ji et al., 2016; Narmada et al., 2013; Yue et al., 2017). TGF-β1 is a pro-fibrotic cytokine that promotes excessive extracellular matrix (ECM) synthesis and deposition, resulting in multi-organ fibrosis (Zhou et al., 2017). Two important downstream regulators of fibrosis are smad2 and smad3, which support TGF-β1-mediated tissue growth (Hu et al., 2018). We therefore verified this pathway in order to comprehend the mechanism of fibrosis brought on by chemotherapeutic ovarian damage.

I3C is a monomer found in the leaves and stems of Brassica plants like broccoli, cauliflower, Brussels sprouts, and cabbage (Bradlow and Zeligs, 2010; Donald et al., 2004). I3C has anti-inflammatory, antioxidant, antiviral, apoptosis-inhibiting, and antitumor properties, according to numerous studies (Khan and Langmann, 2020; Li et al., 2022; Popolo et al., 2017). Furthermore, I3C increases AMPK activation in the heart while decreasing mTOR and ERK1/2 activation, which can effectively prevent and reverse pressure overload-induced myocardial hypertrophy, fibrosis, and dysfunction (Deng et al., 2013). I3C has also been discovered to promote liver fibrosis reversal in vivo by blocking the Inhibitor of κB Kinase α/Inhibitor of κB-α/Nuclear Factor-κB Pathway (Ping et al., 2011). I3C has demonstrated abilities to prevent illness progression and lessen harm in a number of disorders, in conclusion.

However, it is unknown how I3C contributes to ovarian damage brought on by chemotherapy. In this study, we aimed to determine whether I3C is able to alleviate the ovarian injury cause by chemotherapy in mice model and whether it exhibit antitumor properties. Here, we provide evidence that I3C can maintain the primordial follicles reserve and decrease the apoptotic level of granulosa cells in vivo. Also, we found that I3C treatment is capable of reducing ovarian fibrosis and it functions via regulating the TGF-β1/Smad pathway. In vitro study, we demonstrated that I3C inhibit the proliferation, migration and colony formation of cervical cancer cells.

Materials And Methods

Reagents and antibodies

Cisplatin (HY-17394) and Indole-3-carbinol (HY-N0170) was purchased from MCE (New Jersey, USA). TUNEL ApoGreen Detection Kit (40307ES20), the 1st Strand cDNA Synthesis Kit (11141ES60) and Hieff
qPCR SYBR Green Master Mix(11201ES08) were purchased from YEASEN Biotechnology (Shanghai, China). Cell Counting Kit-8 (CT0001) was bought from SparkJade (Shandong, China). The primary antibodies used in this study were as follows: anti-AMH (620222), anti-Bax (380709), anti-Bcl2 (381702), Collagen I (501352), anti-alpha-SMA (380653), anti-TGF beta 1 (346599), anti-Smad2 (200790), anti-phospho-Smad2 (Ser250, R26361), anti-Smad3 (R25743), anti-phospho-Smad3 (Ser423/425, 380775), anti-phospho-H2A.X(Ser139, 381558), anti-beta tubulin (380628), anti-GAPDH (380626) were purchased from Zen BioScience (Chengdu, China). Rabbit anti-PCNA polyclonal antibody (bs-2007R) was obtained from Bioss (Beijing, China)

**Animals**

ICR female mice were bought from the Experimental Animal Center of Anhui Medical University at ages 3 weeks (n = 30) and 8 weeks (n = 18). All animals were kept in specialized, pathogen-free environments with a constant temperature of 22°C, a humidity range of 50–60%, and 12 hours of light each day. Prior to the studies, the animals underwent a 3-day acclimatization period during which they had unrestricted access to food and water. The Experimental Animal Ethics Committee of Anhui Medical University approved all experimental methods, which were carried out in compliance with international ethical standards (approval number).

**Group and treatment**

Female ICR 8-week mice were divided into control (Ctr), cisplatin (Cis), and cisplatin + I3C (Cis + I3C) groups at random. Mice in the control group received daily intraperitoneal injections of PBS for 7 days; the cisplatin group received daily intraperitoneal injections of cisplatin (2 mg/KG) for 7 days to construct an ovarian injury model (Lu et al., 2020); the cisplatin + I3C group received daily intraperitoneal injections of I3C (30 mg/kg) at 3-hour intervals and intraperitoneal injections of cisplatin (2 mg/KG) for 7 days. All mice were sacrificed once the prescribed doses had been administered, and the ovaries were collected and weighed on an electronic balance.

**Ovarian hematoxylin eosin staining and follicle count**

Ovaries were fixed for 24 hours in 4% paraformaldehyde, paraffin embedded, serially sectioned at a thickness of 5um, then stained with hematoxylin and eosin (H&E). According to the accepted criteria set by Pedersen and Peters, follicular phases are categorized. primordial or primary follicles are defined as oocytes surrounded by a single layer of flat or cuboidal granulosa cells. Secondary follicles are defined as oocytes surrounded by more than one layer of cuboidal granulosa cells without a visible lumen. Whereas antral follicles are defined as follicles with a clear luminal gap and containing follicular fluid. To determine the total number of follicles at each stage, follicle counts were done on the ovaries of 5 mice in each group, and only follicles with visible oocyte nuclei were counted every 5th slices.

**Estrous cycle examination**

Vaginal smears were performed every day at 8 a.m. for 10 days. The vaginal fluid was then smeared on slides and stained with H&E staining. Under a light microscope, the estrous cycle was separated into four
phases: proestrus (P), estrus (E), metestrus (M), and diestrus (D), which typically lasted for 4–5 days, based on the proportion of keratinized epithelial cells, nucleated epithelial cells, and polymorphonuclear leukocytes (Ekambaram et al., 2017).

**Western blot analysis**

For Western blotting analysis, Radio-immunoprecipitation assay (RIPA) buffer containing enzyme-inhibitor was used to lyse the ovarian tissues or Caski and Hela cell lines. Protein samples were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and blocked for 1 hour at room temperature with 5% skim milk diluted in TBST (TBS-0.1% Tween-20). Subsequently, the PVDF membranes were incubated overnight at 4°C with a variety of antibodies, including anti-AMH (1:1000), anti-α-SMA (1:2000), anti-collagen I (1:1000), anti-TGF-β1 (1:1000), anti-Smad3 (1:1000), anti-p-Smad3 (1:1000), anti-Smad2 (1:1000), anti-p-Smad2 (1:1000), anti-Bax (1:1000), anti-Bcl2 (1:1000), anti-PCNA (1:1000), anti-GAPDH (1:2000) and anti-Tubulin (1:1000). Membranes were cleaned three times with TBST before being incubated for an hour with a secondary IgG antibody conjugated to horseradish peroxidase (HRP) and diluted in 5% skim milk. ECL Prime Western Blotting detection reagent was utilized to find each protein's expression. Using the CS Analysis System (5200, Tanon, Shanghai, China), the target proteins' luminous bands were discovered.

**Quantitative realtime PCR**

Total RNA was isolated from ovarian tissue using an RNA extraction kit (Yeasen, 19221ES50). A total of 2.5ug RNA was utilized as the template for the reverse transcription procedure. The primers used are indicated in the Table 1. After that, real-time quantitative PCR was carried out using SYBR Green I Masser (Roche, Switzerland, Germany). Finally, the $2^{-\Delta\Delta Ct}$ technique was used to analyze relative gene expression.

**Table 1**  
List of qRT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer(5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fshr</td>
<td>CTTTCCTCTTGGTCTCCTTG</td>
<td>CTCGGTACCTTGCTATCTTG</td>
</tr>
<tr>
<td>Lhr</td>
<td>CGCCCACTATCTCTCACTCA</td>
<td>GACAGATTGAGGAGTGTCAAA</td>
</tr>
<tr>
<td>Col1a2</td>
<td>GTAACTTCGTGCCTAGCAA</td>
<td>CTTTGTCGAATACTGAGCAG</td>
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<tr>
<td>α-SMA</td>
<td>GTCCCCAGACCTCAGGGAGTA</td>
<td>TCGGATACCTAGCGTCAGGA</td>
</tr>
<tr>
<td>Tgf-β1</td>
<td>GACATGCTGGCTTCTAGGC</td>
<td>GCCTTAGGAAAGAGATGCTG</td>
</tr>
<tr>
<td>Smad2</td>
<td>ATGTCTGCTACTGGCCATTC</td>
<td>AACGTCCTGTTTTCTTTAGCTT</td>
</tr>
<tr>
<td>Smad3</td>
<td>CACGCAGAAGCTGAAACC</td>
<td>GGCGAAGTAAACAGTGAGGA</td>
</tr>
</tbody>
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**Immunohistochemistry**
First, paraffin-embedded ovarian tissue sections were dewaxed and rehydrated before being immersed in a sodium citrate solution (pH = 6.0) for 20 minutes for high-pressure antigen repair, followed by dropwise closure with an endogenous peroxidase blocker for 10 minutes. Tissue sections were then incubated at 4°C overnight with anti-AMH (1:50), anti-α-SMA (1:200), anti-collagen I (1:200), anti-TGF-β1 (1:200), anti-p-Smad3 (1:200), anti-p-Smad2 (1:200), anti-Bax (1:200), and anti-H2AX (1:200), washed three times with phosphate-buffered saline, and incubated for 40 minutes with HRP-coupled goat anti-rabbit IgG secondary antibody. Finally, 3,3-diaminobenzidine tetrahydrochloride (DAB) was used and observed under the microscope until yellow positive cells appeared. The coverslips were sealed and photographed under the microscope for observation, and Image J software was used for computational analysis.

**TUNEL Staining**

TUNEL staining was performed using the TUNEL ApoGreen Detection Kit. To allow permeabilization, ovarian tissues were dewaxed and rehydrated before being incubated in DNAase-free Proteinase K (20ug/ml) solution at room temperature for 20 minutes. This was followed by a 60-minute incubation at 37 degrees using a TDT buffered system while avoiding light. The nuclei were observed by re-staining with Hochst33342 (1:500). Finally, the apoptotic signal was observed under fluorescence microscopy.

**Cell culture**

The human cervical cancer cell lines Hela and Caski were cultured in DMEM and 1640 media, respectively, with 10% fetal bovine serum and 1% penicillin/streptomycin. Incubation conditions were 37°C and 5% CO2.

**Cell viability assay**

Cell viability was measured using Cell Counting Kit-8 (CCK-8). Hela cells or Caski cells was seeded in 96-well plates at a density of 1000 cells per well, and treated by I3C at a concentration gradient of 20 ug/ml, 40 ug/ml for 4 days. CCK-8 assay reagent (10 µL) was added to each well containing 100 µL medium, and then incubated in the dark for 2 hours at 37°C. The OD values were measured using microplate spectrophotometer at 450 nm every day.

**Wound healing assay**

Each group of cells was cultured in 6-well plates, scratched vertically with a 100ul pipette, then washed three times with PBS and photographed under microscope, with the width of the scratch recorded and counted as 0 hours. Cells were treated with DMSO or I3C (40 ug/ml) for 24 hours. Pictures were obtained and quantitative analysis was carried out using ImageJ software.

**Colony formation assay**

A total of 1000 Hela cells or Caski cells were cultured in 6-well plates per well. After 24 hours, the cells were incubated with DMSO or I3C (10ug/ml, 20ug/ml, and 40ug/ml). The cells were fixed with methanol for 10 minutes after 12 days of incubation. And then cells were stained with 0.5% crystalline violet solution for 10 minutes.
Statistical analysis

The experimental data were analyzed statistically using GraphPad Prism 9.0, and the mean and standard error were shown for each experiment. At least three independent samples from each experiment were used, and the experiment was duplicated at least three times. Samples from two groups were compared using unpaired t-tests, while samples from more than two groups were compared using one-way ANOVA. Statistical significance was defined as P values less than 0.05.

Results

I3C treatment restored the ovarian function in mice model of ovarian injury

To evaluate the effect of I3C on cisplatin-induced ovarian injury mice model, we firstly weighed the body weight and ovarian weight of each group and calculated the ovarian index. And results show that body weight, ovarian weight and ovarian index were considerably reduced in the cisplatin alone treated group compared to the control group, whereas these indices were improved in the cisplatin and I3C co-treated group (Fig. 1a-d). Then, by analyzing the estrous cycle, we found that the mice of Cis group exhibited irregular estrous cycles with a shorter estrous phase and a longer diestrous phase, compared to control group and Cis + I3C group (Fig. 1e-i).

Follicle counting revealed that the number of primordial follicles was significantly reduced in mice treated with cisplatin compared to the control group, whereas the number was recovered in mice co-treated with I3C and cisplatin. As shown in Fig. 2a-b, the number of activated follicles, especially primary follicles, was significantly increased compared to the control group, indicating that cisplatin treatment promoted the transition from primordial follicles to primary follicles. However, I3C treatment can inhibit the over-activation of primordial follicles to maintain the primordial follicle reserve. Numerous studies have demonstrated that anti-müllerian hormone (AMH) inhibits primordial follicle activation, and our results revealed that I3C was able to restore the decreased AMH protein levels caused by cisplatin treatment (Fig. 2c,d). We also found cisplatin significantly increased the expression levels of Fshr and Lhr mRNA, but I3C treatment was capable of restored the levels of Fshr and Lhr (Fig. 2e,f). In conclusion, these findings indicated that I3C administration recovered ovarian function in chemotherapy mice.

I3C suppressed the cisplatin-induced apoptosis in ovarian tissue

The impact of I3C on cisplatin-induced apoptosis was then examined in mice ovarian tissues. Western blot results revealed that cisplatin treatment elevated the expression level of the Bax and down-regulated the ratio of Bcl-2/BAX. In contrast, I3C therapy reduced the elevated amount of Bax expression and decreased the ratio of Bax/Bcl2 expression (Fig. 3a-c). The IHC staining results also confirmed that Bax
levels were significantly lower in the Cisplatin + I3C group than in the cisplatin group, and that Bax-positive cells were primarily concentrated in granulosa cells of growing follicles (Fig. 3d-f). When compared to the cisplatin group, I3C diminished the expression level of γH2AX, another important regulator of the cellular response to DNA damage (Fig. 3d, f). TUNEL assay revealed that the number of TUNEL-positive cells, particularly granulosa cells, increased significantly in the cisplatin group while being substantially decreased by I3C treatment (Fig. 5g,h). In conclusion, I3C reduced cisplatin-induced apoptosis in mouse ovarian tissue, particularly in granulosa cells.

Effect of I3C in cisplatin-induced ovarian fibrosis in mice

To further investigate the protective effect of I3C on mice ovary undergoing chemotherapy, we detected fibrosis-related markers, α-SMA and Collagen I, by IHC staining, and the results revealed that their positive cells were mainly located in the ovarian mesenchyme, and cisplatin injection alone significantly increased the levels of α-SMA and Collagen I, whereas the combined injection of cisplatin and I3C significantly decreased the levels of α-SMA and Collagen I (Fig. 4a-c). Furthermore, results of WB and qRT-PCR confirmed similar results (Fig. 4d-h). These findings suggest that I3C can reduce cisplatin-induced ovarian fibrosis.

I3C alleviates cisplatin-induced ovarian fibrosis through TGF-β1/Smads signaling pathway

The TGF-β1/Smad pathway is currently recognized as the primary route causing fibrosis(Zhou et al., 2017). We explored this pathway in ovarian tissues to figure out how I3C prevents ovarian fibrosis in chemotherapy mice. The TGF-β1, p-Smad2, and p-Smad3 levels in the ovarian tissues of the cisplatin group were higher than those in the control group, according to the IHC staining result, however the levels in the Cisplatin + I3C group were dramatically downregulated in comparison to cisplatin alone (Fig. 5a-d). Notably, granulosa cells were the primary location of the TGF-β1, p-Smad2 and p-Smad3 positive cells. WB results showed that the levels of Smad2, Smad3 and their phosphorylated proteins were reduced in the Cisplatin + I3C group compared with the cisplatin group (Fig. 5e-j). We also further confirmed the changes of mRNA levels of Tgf-β1, Smad2 and Smad3 among the groups by qRT-PCR. And the result showed that the mRNA levels of these genes were significantly lower in the Cisplatin + I3C group compared with the cisplatin group (Fig. 5k-m). These results suggest that I3C can downregulate the activity of TGF-β1/Smad pathway to attenuate cisplatin-induced ovarian fibrosis.

Effect of I3C in cervical cancer cells

The anti-tumor activity of I3C has been reported in the literature(Popolo et al., 2017). In order to assess whether I3C can inhibit the growth of gynecological tumors, we performed the cell proliferation assay by CCK-8, clone formation assay, and wound healing assay on using two cervical cancer cell lines, Hela and Caski cells. And as shown in Fig. 6a, b I3C treatment was able to suppressed the proliferation of Hela and caski cells in a dose-dependent manner compared to the control group. Moreover, I3C dramatically
decreased the expression level of proliferating cell nuclear antigen (PCNA) in both cell lines, according to western blot data (Fig. 6c-f). And the result of the colony formation assay revealed a considerable dose-dependent decrease of the capacity of Hela and caski to form colonies (Fig. 6g, j). The wound healing assay demonstrated that I3C treatment significantly reduced the migratory ability of Hela and Caski cells (Fig. 6k-n). Taken together, these results indicate that I3C have anti-tumor potential.

Discussion

Due to the susceptibility of ovarian tissue to chemotherapy, it is now necessary to consider how to effectively maintain and preserve fertility throughout oncology treatment. Finding substances that can shield ovarian tissues from chemotherapy drugs is therefore urgently needed. I3C, however, has been demonstrated in numerous trials to lessen injury and prevent disease in a number of organs. I3C inhibits the proliferation and neointima formation of cultured vascular smooth muscle cells in a carotid artery injury model via the Akt/GSK3β pathway to prevent vascular value-added disease (Guan et al., 2013). Nevertheless, it has not been reported whether I3C can alleviate ovarian damage caused by chemotherapy and protect fertility. In this study, we discovered for the first time that combining I3C with cisplatin could alleviate cisplatin-induced ovarian damage, and we also investigated the mechanism by which I3C alleviates ovarian damage in chemotherapy mice.

We established an ovarian injury model by intraperitoneal injection of cisplatin. Based on this model we have observed that I3C can restore ovarian index and regulate estrous cycle in mice. It has long been accepted that ovarian reserve is not restorative and that women develop primordial follicular reserve during fetal life (Lew, 2019). In order to maintain the balance of their reproductive lifespan, females are physiologically conditioned to rationalize the use of follicular reserve through the orderly regulation of primordial follicular activation (Hansen et al., 2008). Numerous investigations have demonstrated that cisplatin treatment causes the loss or overactivation of primordial follicles, which lowers the ovarian reserve (Chang et al., 2015). Our results are consistent with previous findings that the number of primordial follicles decreased significantly and the number of growing follicles increased significantly in the cisplatin group, which was reversed with the use of I3C, suggesting that I3C can delay the cisplatin-induced decline in ovarian reserve. AMH, which is secreted by granulosa cells of growing follicles and inhibits the activation of primordial follicles, is another indicator of ovarian reserve (Durlinger et al., 1999; Sonigo et al., 2019). Cisplatin downregulated AMH expression in our study, but AHM expression levels recovered after cisplatin co-treatment with I3C (Fig. 2c, d). Furthermore, FSHR and LHR have been linked to a regulatory role in folliculogenesis (Khalil et al., 2022). FSHR and LHR were found to be higher in primary follicles than in primordial follicles in a previous study(Zheng et al., 1996). We discovered that I3C treatment reduced the levels of Fshr and Lhr mRNA in ovarian tissues (Fig. 2e, f), and we hypothesized that this reduction was related to I3C's ability to inhibit primordial follicles over-activation.

Although we have gained a better understanding of the mechanisms underlying chemotherapy-induced ovarian damage in women of childbearing age in recent years, we still do not know which mechanisms are the most important in this process. Notably, while the vast majority of current protective agents focus
solely on follicular effects, the stromal environment of the ovary also contributes to follicular health. Ovarian cortical fibrosis has been shown to be an important additional mechanism of chemotherapy-induced ovarian functional impairment (Meirow et al., 2007). The most common events in fibrotic disease have been reported to be EMT and ECM synthesis, with type I collagen being the most abundant component of the ECM and widely expressed in the ovary (Briley et al., 2016; Wormstone et al., 2009). Our results indicated that I3C reversed the cisplatin-induced increase in Collagen I and the EMT marker, α-SMA. To put it another way, I3C was able to slow the progression of cisplatin-induced ovarian fibrosis. The TGF-β1/Smad signaling pathway is important in ovarian fibrosis and has been extensively researched, particularly in polycystic ovary syndrome (Takahashi et al., 2017; Wang et al., 2018; Zhou et al., 2022). Once activated, ligand binding results in the formation of the receptor heterodimeric complex TGFβRII/TGFβRI, which then recruits and activates Smad2 and Smad3, phosphorylates the Smad2/3 complex with Smad4, and translocates to the nucleus to activate downstream target genes (Moustakas and Heldin, 2009). Interestingly, and in line with prior findings, we discovered that TGF-β1 and p-Smad2/3 were expressed in granulosa cells of mouse ovaries. We established that I3C’s ability to decrease ovarian fibrosis was reliant on suppressing the TGF-β1/Smad signaling pathway, preventing the ovarian damage caused by cisplatin.

In this study, we confirmed that I3C can play a protective role in chemotherapy-induced ovarian damage, so will it interfere with the anti-tumor efficacy? Several studies have confirmed the antitumor activity of I3C. For example, Lee et al. first found that in myc-driven or Pten heterozygous mice, I3C inhibited the reactivation of Pten by WWP1, leading to an effective inhibition of tumorigenesis driven by the PI3K-AKT pathway (Lee et al., 2019). By suppressing the EMT process, I3C also reduced the migration of breast cancer cells (Ho et al., 2013). Our findings even shown that I3C was able to prevent cervical cancer cells from proliferating and migrating in vitro. This establishes the groundwork for additional research into the use of I3C to safeguard ovarian function.

**Conclusion**

In summary, we provide convincing evidence that I3C inhibits TGF-β1/Smad2/3 signaling pathway activity, reduces ovarian fibrosis, protects against cisplatin-induced ovarian damage, and has antitumor properties. Therefore, I3C could be a promising candidate drug for preserving fertility in female cancer patients.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Hospital Ethics Committee of the First Affiliated Hospital of Anhui Medical University

**Consent for publication**
Our manuscript follows the authorship statement of ethical standards for manuscripts submitted to Molecular Medicine.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare no competing financial interests.

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Author contribution

FZ, FL, HQ, SW contributed equally to this work. The study was designed and wrote by FZ, HL, XL. FZ, FL, HQ, SW performed research; JC, ZH, YK analyzed the data. All authors read and approved the final manuscript.

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treatment. BIOMED PHARMACOTHER 101, 670-681.


Figures
Figure 1

Detection of ovarian index and analysis of vaginal smear.

(a) Representative photograph of General appearance of each group ovaries. (b) Body weight, (c) ovarian weight, and (d) ovarian index (ovarian weight/ body weight) of the three groups of mice. (e) Representative H&E staining images of mouse vaginal smear at different stages of the estrous cycles. Scale bars, 50 µm. (f) Relative proportion of each estrous cycles in the four groups of mice. P, proestrus; E, estrus; M, metestrus; D, diestrus. (g-i) Typical changes of estrous cycles in the three groups of mice. Data are presented as mean ± s.e.m, n = 6 mice, *P < 0.05, **P < 0.01, ***P < 0.001. ****P<0.0001. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.
Figure 2

Follicle counting and detection of biomarker of follicular development. (a) Representative images of H&E-stained ovarian morphology. Scale bars, 100 µm. (b) The number of primordial follicles (PMFs), primary follicles (PFs), secondary follicles (SFs) and antral follicles (AFs) per ovary in each treatment group was shown in the column. (c) Western blot for AMH expression in each group of ovarian tissue. Representative blots from one of four independent experiments were shown. n= 4 biologically independent replicates. GAPDH was used as a loading control. (d) Quantitative analysis of AMH protein expression using Image J software. (e-f) Relative levels of FSHR (e) and LHR mRNA (f) in ovarian tissues of each group. n=6 biologically independent replicates. For bar graphs in (b, d-f) data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.****P<0.0001. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.
Apoptosis of granular cells caused by cisplatin was inhibited after I3C treatment.

(a) Bax and Bcl-2 protein expression levels were measured by Western blot in the ovarian tissues of each group. n= 4 biologically independent replicates. GAPDH was used as a loading control. (b-c) Quantitative analysis of the expression levels of Bax (b) and Bax/Bcl2 (c). (d) Representative IHC results of Bax and α-SMA.
γH2AX in ovarian tissues of each group. n= 3 biologically independent replicates. Scale bar, 100 µm. (e-f) Quantification of the positive area for Bax (e) and γH2AX (f). (g) Apoptotic cells in ovarian tissue were detected using TUNEL assay. Green represents apoptotic signals. The nuclei were stained with Hoechst and fluoresced blue. n= 3 biologically independent replicates. Scale bars, 200 µm. (h) Quantitative analysis of fluorescence intensity of apoptotic cells. For bar graphs in (b-c, e, f, h) data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.
Figure 4

Staining and detection of markers of fibrosis.

(a) The immunohistochemistry results of α-SMA and collagen I in ovarian tissues in each group. n= 3 biologically independent replicates. Scale bars, 100 µm. (b-c) Quantification of α-SMA (b) and collagen I (c) positive areas. (d) Detection of collagen I and α-SMA by Western blot in each group of ovarian tissues. n=4 biologically independent replicates. (e-f) Quantitative analysis of collagen I (e) and α-SMA (f) protein expression. (g-h) Relative levels of Col1a2 (g) and α-SMA mRNA (h) in ovarian tissues of each group. n=6 biologically independent replicates. For bar graphs in (b-c, e-h) data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. ****P<0.0001. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.
TGF-β/smads pathway was down-regulated by I3C.

(a) The immunohistochemistry results of p-Smad2, p-Smad3 and TGF-β1 in ovarian tissues of each group. n= 3 biologically independent replicates. Scale bars, 100 µm. (b-d) Quantitative analysis of p-Smad2 (b), p-Smad3 (c) and TGF-β1 (d). (e) Protein level of p-Smad2, Smad2, p-Smad3, Smad3, and...
TGF-β1 in each group of ovarian tissues. n = least 3 independent biological replicates. GAPDH was used as the loading control. (f-j) Quantitative analysis of p-Smad2, Smad2, p-Smad3, Smad3, and TGF-β1 protein expression levels. (k-m) The RNA expression of TGF-β1, Smad2 and Smad3 by qRT-PCR analysis in each group. GAPDH was used as an internal control. n=6 biologically independent replicates. For bar graphs in (b-d, f-m) data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.

Figure 6

I3C exhibits the ability of anti-tumor in vitro.

(a-b) viability of Caski (a) and HeLa cells (b) after treatment with different concentrations of I3C for 0, 24, 48, 72 and 96 hours. (c-f) PCNA protein expression levels in Caski (c) and Hela (e) cells after I3C
treatment for 24 hours and its statistical analysis results (d, f). (g) Colony formation assay of CasKi and (i) HeLa cells treated with I3C at various concentrations. (h) statistical results of CasKi and (j) HeLa cell colony formation assay. (k) Wound healing assays of I3C-treated CasKi and (m) HeLa cells at different times and (l, n) their statistical analysis. For bar graphs in (a, b, d, f, h, j, l, n) data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. #P <0.0001. Statistical comparisons were performed using unpaired Student's t-tests.