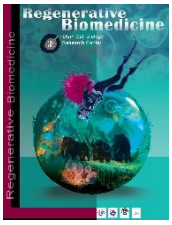

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Research Article

Carvacrol Regulates the Expression of Genes Involved in the TGF-β/Smads Signaling Pathway in a Rat Model of POI

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Abstract

Premature ovarian insufficiency (POI), an abnormal ovarian function has been documented in females. Besides, the clinical treatment of POI patients is not completely satisfactory. The blunting effects of Carvacrol in Cyclophosphamide (CTX)-induced Premature Ovarian Insufficiency (POI) were studied in rats.

Firstly, animals received CTX for two consecutive weeks to induce the POI model. Once the POI model was confirmed, the intervention group was injected with 80 mg/kbw Carvacrol for 5 days. Four weeks after the last injection, serum samples were collected for hormonal evaluation, and tissue samples were isolated and analyzed for histopathology evaluation and the expression of TGF-β/Smad pathway genes.

Data showed CTX injection altered FSH and LH hormones ($p>0.05$). Following Carvacrol injection, FSH and LH levels were diminished ($p<0.05$). Histopathological evaluation revealed the increase of morphologically healthy follicles ($p<0.05$). The transcription of Smad pathway genes such as Smad 2,-4, and TGF-β1 were induced after CTX injection related to control rats ($p>0.05$). However, after Carvacrol intervention, the gene expression was modulated, and a statistically significant decrease in Smad 6 expression was observed ($p<0.05$).

Carvacrol administration can restore sex hormone levels, improve follicular morphology, and modulate the expression of Smad pathway genes.

Keywords: Premature Ovarian Insufficiency, Monoterpenoid phenol, Fibrosis, Ovarian Regeneration, Reproduction

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Introduction

POI coincides with abnormal ovarian function before 40 years old. This leads to the loss of menstruation and abnormal growth of follicles, resulting in the reduction of follicle production and infertility (1, 2). An important indicator of POI is the increase in gonadotropins, particularly the FSH hormone, and the decrease in estrogen levels (3). The prevalence of premature ovarian failure is increasing annually in developing countries. Studies have shown that the prevalence of POI ranges from 0.9 to 2% in several communities (4). Approximately 20% of cases are attributed to genetic factors and abnormalities, while the remaining 80% are related to autoimmune, endocrine, mitochondrial, iatrogenic diseases, infection, and environmental factors (5).

Besides to hormone therapy (6), novel clinical approaches have emerged for POI patients. These include treatment with various types of stem cells (7, 8), ovarian freezing (9), and in vitro fertilization (IVF) (10). Cell-based therapies using stem cells have been touted as a potential *de novo* and alternative treatment approach for ovarian tissue recovery in POI (11). Stem cells can improve the functions of injured ovarian tissue, including sex-related hormones and ovarian follicle number at different stages, and modulate the expression of genes in animal models of POF (12). However, the application of several stem cell lineages may have safety issues and limitations. Additionally, due to the complex pathogenesis of POI, the clinical treatment is not completely satisfactory, and ongoing studies are being conducted (13). Carvacrol or Cymophenol (C6H3C3H7) is a natural phenol and monoterpenoid derivative found in various plant species such as thyme, black cumin, wild bergamot, and pepper (14). Numerous studies have shown that Carvacrol exhibits antioxidant, bactericidal, fungicidal, tumoricidal, anti-inflammatory, antidiabetic, antispasmodic, antimicrobial, immune system modulator, and growth stimulator properties (15). Emerging studies have demonstrated that Carvacrol can alter the expression of TGF-β/Smads pathway genes in the kidney and liver tissues of mice, leading to modulation of the gene expression pathway involved in tissue hemostasis and fibrosis (16, 17). Other studies have reported that Carvacrol exhibits antioxidant properties and reduces inflammation, fibrosis, and apoptosis in various tissues. Previous studies have also shown the role of Carvacrol in maintaining ovarian reserve (18). Despite these studies, there have been no reports investigating the effects of Carvacrol on cyclophosphamide-induced POI via the TGF-β/Smad signaling pathway. Given the biological importance of Carvacrol, we aimed to assess the biological properties of Carvacrol on ovarian tissue regeneration.

Materials and methods

Animal ethics

The study was performed using 20 female Wistar rats weighing an average of 300 to 400 grams. These rats were obtained from Tehran's Med Zist Company and transferred to the animal center of our institute. The rats were housed in standard conditions with free access to drinking water and chewable pellets. All steps of this project were approved by the Animal Care and Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.AEC.1402.053).

Induction of the rat model of POI

One week after animal accommodation, the rats were intraperitoneally injected at day first with 200 mg/kg BW (CTX) and 8 mg/kg BW Cyclophosphamide from day 2nd to 14th to induce the POI model. After 20 days, to confirm the POI condition, 3 rats from each group were randomly selected and euthanized. Tissue samples were taken for gene expression and histopathology evaluation, and blood samples were taken for sex hormone evaluation. After confirming, the POI rats were randomly divided into control POI and Carvacrol groups.

Carvacrol injection

Twenty-one days after the last injection of CTX, 80 mg/kg Carvacrol (Sigma, Cat No: 499-75-2) was intraperitoneally injected for 5 days in the intervention group. Carvacrol was mixed with alcohol in a ratio of 1:5 as a solvent (19). One month after the last day of Carvacrol injection, the samples were euthanized for histopathology, hormonal, and gene expression studies.

Histopathological analysis

Four weeks after Carvacrol injection, the animals were euthanized and left ovaries were fixed with 4% formalin solution. Paraffin-embedded blocks were cut into 5 µm thickness using a microtome tool from Leica and stained with H&E staining solution (20). The number/quality of follicles in growth stages and the presence of corpus luteum (CL) were evaluated using a light microscope.

Hormonal evaluation

Using ELISA, systemic contents of FSH, LH, and E2 were measured (FSH: 4-096-334, LH: 96-0234, E2: 4925-300A; Monobind). Blood samples were centrifuged at 400xg for 20 minutes to collect serum, which was then stored at freezing temperature until use.

Gene expression analysis

Right ovarian tissues were subjected for analysis of the Smad2, 4, 6 and TGF-β1 expressions using specific primer pairs for each gene according to our previously published data (21). The specificity of the reactions was assessed by analyzing the melting curves. Expression levels were calculated using the $2^{-\Delta\Delta ct}$ method related to β-actin. (The sequences of utilized primers are presented in table 1)

Statistical Analysis

Data [mean ± SEM] were analyzed (GraphPad Prism ver. 8.0.1 software) using One-way analysis of variance with post-hoc test (Fisher's Least Significant Difference, LSD) and Student's t-test. $P<0.05$ were considered significant.

Results

Successful induction of POI by CTX administration

To confirm the effects of animal modeling following CTX injection, histopathological evaluation was performed in ovarian follicles 20 days after the last injection (Fig. 1A). We observed a decrease in the number of morphologically healthy follicles in the POI group compared to the control group ($p<0.05$; Fig. 1B). In contrast, we observed general atresia of follicles in the POI group, characterized by vacuolization, disintegration

of cumulus cells, and detachment of granulosa cells in all developing follicles, compared to the healthy rats ($p<0.05$, Fig. 1C). Furthermore, we observed a decrease in corpus luteum (CL) in the POI group compared to the control group ($p<0.01$; Fig. 1D). Our findings indicate that administration of CTX leads to morphological conditions similar to POI.

Carvacrol intervention increased healthy follicles

Four weeks after the last administration of CTX, we examined morphologically healthy and atretic follicles under a light microscope (Fig. 2A). Our findings showed that the administration of CTX increased the number of healthy follicles in the ovarian tissue of rats compared to the POI control group ($p<0.05$) (Fig. 2B). Interestingly, the number of atretic follicles after CTX administration was reduced compared to the POI control group ($p<0.05$) (Fig. 2C). We also observed an increase in CL in the Carvacrol-treated rats compared to the POI rats, although no significant differences were obtained (Fig. 2D).

Carvacrol intervention modulated the serum levels sex hormones

We evaluated the FSH, LH, and E2 hormones pre- and post-CTX administration. Data showed FSH and LH hormones were reduced in the Carvacrol-treated rats related to POI subjects, however this difference was not statistically significant. In addition, the level of FSH and LH hormones decreased after the intervention ($p<0.05$). Regarding E2 levels, this hormone was declined before the intervention and increased after Carvacrol injection related to POI rats. These changes did not reach significant levels (Fig. 3).

Carvacrol regulated the expression of TGF-β/Smads signaling pathway

Real-time PCR results showed that the expression of Smad2, Smad4, and Tgf-β1 genes increased after CTX injection. We noted that the administration of Carvacrol modulated the transcription level, although no statistically significant differences were observed. Additionally, we found that there was no appreciable change in the Smad6 gene before the intervention. However, the administration of Carvacrol significantly reduced the expression level of the Smad6 gene ($p<0.05$; Fig. 4).

Discussion

CTX is a conventional therapeutic drug used for chemotherapy. It is an alkylating drug widely used as an antitumor and immunosuppressive medication. However, along with its therapeutic benefits, CTX disrupts ovarian function and sex hormone secretion, posing the highest risk of POI (8, 22, 23). Various studies have confirmed that CTX chemotherapy causes loss of ovarian reserve and disturbance in folliculogenesis (24, 25). Inducing the POI model with CTX can increase atretic follicles and decrease healthy follicles (primary, primordial, secondary, and antral) (26-29). Our results confirm that CTX maximizes atretic follicles, consistent with previousstudies. Therefore, CTX was successfully used to induce the rat POI model.

One important indicator of POI is the FSH hormone. In a normal state, the FSH level is low. As mentioned, CTX disrupts sex hormones and leads to an increase in FSH and

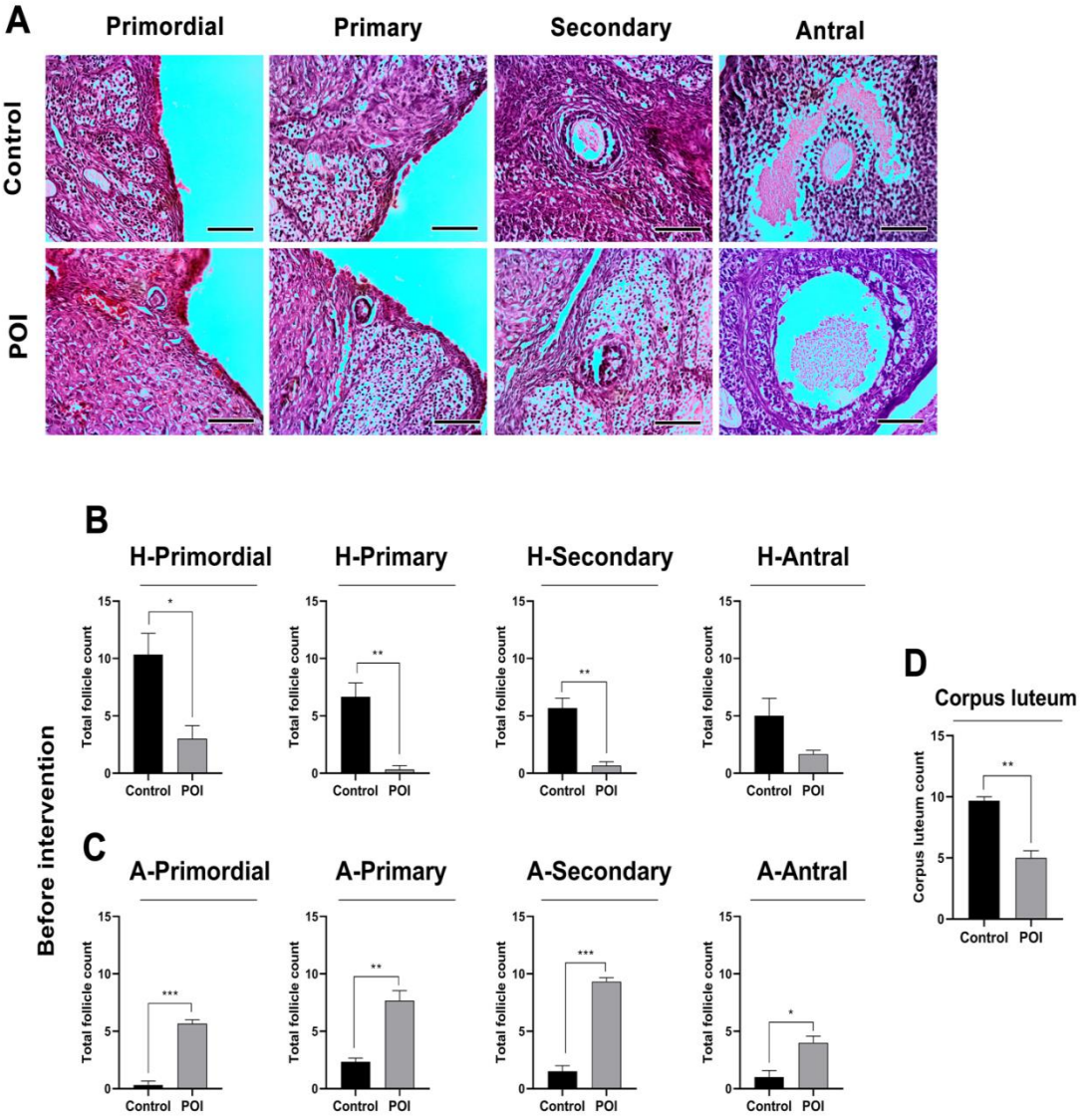

Figure 1. Morphological evaluation of ovarian tissue before intervention, H&E staining (A), the number of healthy follicles in different stages of primordial, primary, secondary and antral growth before intervention (B), the number of atretic follicles in different stages of growth before intervention (C), total corpus luteum count before intervention (D). A=arthritic, H=healthy, (Scale bar=200 µm), *p<0.05; **p<0.01 and ***p<0.001 (n=3).

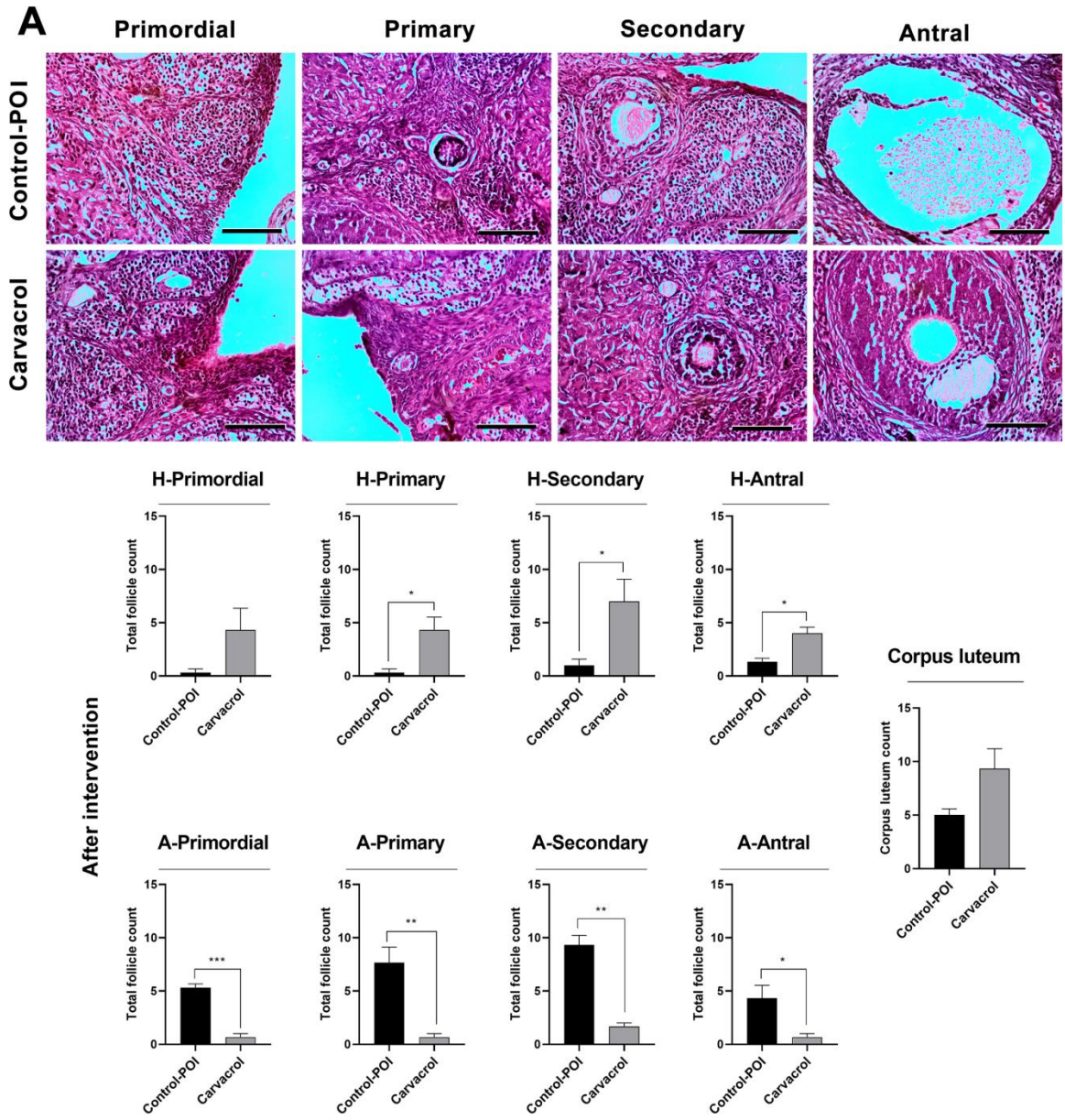

Figure 2. Morphological evaluation of ovarian tissue after intervention, H&E staining (A), different types of atretic follicles count after intervention (B), the number of healthy follicles in different stages of growth after intervention (C), total corpus luteum count after intervention (D). (A=arthritic), (H=healthy), (Scale bar=200 μm), *p<0.05; **p<0.01 and ***p<0.001 (n=3).

Table 1. Primers sequences used for Real time PCR

Genes	Sequence (5' → 3')	Annealing T (˚C)	Reference
Rn-SMAD2	F: TCCATCGAACTCGGAGAGGT	60	(21)
NCBI ref: NM-001277450-1	R: ATACAAGCGCACTCCCCTTC		
Rn-SMAD4	F: GCAACCCCCATCACCTTAGT	60	(21)
NCBI ref: NM-019275.3	R: CATCGGAGGAAGGTACAGCG		
Rn-SMAD6	F: CGCCTCTATGCGGTGTATGA	60	(21)
NCBI ref: NM-001109002.2	R: AGCAGGATGCCAAAACCGAT		
Rn-TGF-β1	F: TCCATGACATGAACCGACCC	60	(21)
NCBI ref: NM 021578.2	R: TGCCGTACACAGCAGTTCTT		
Rn-β-actin	F: TGACAGGATGCAGAAGGAGA	60	(21)
NCBI ref: NM_031144.3	R: TAGAGCCACCAATCCACACA		

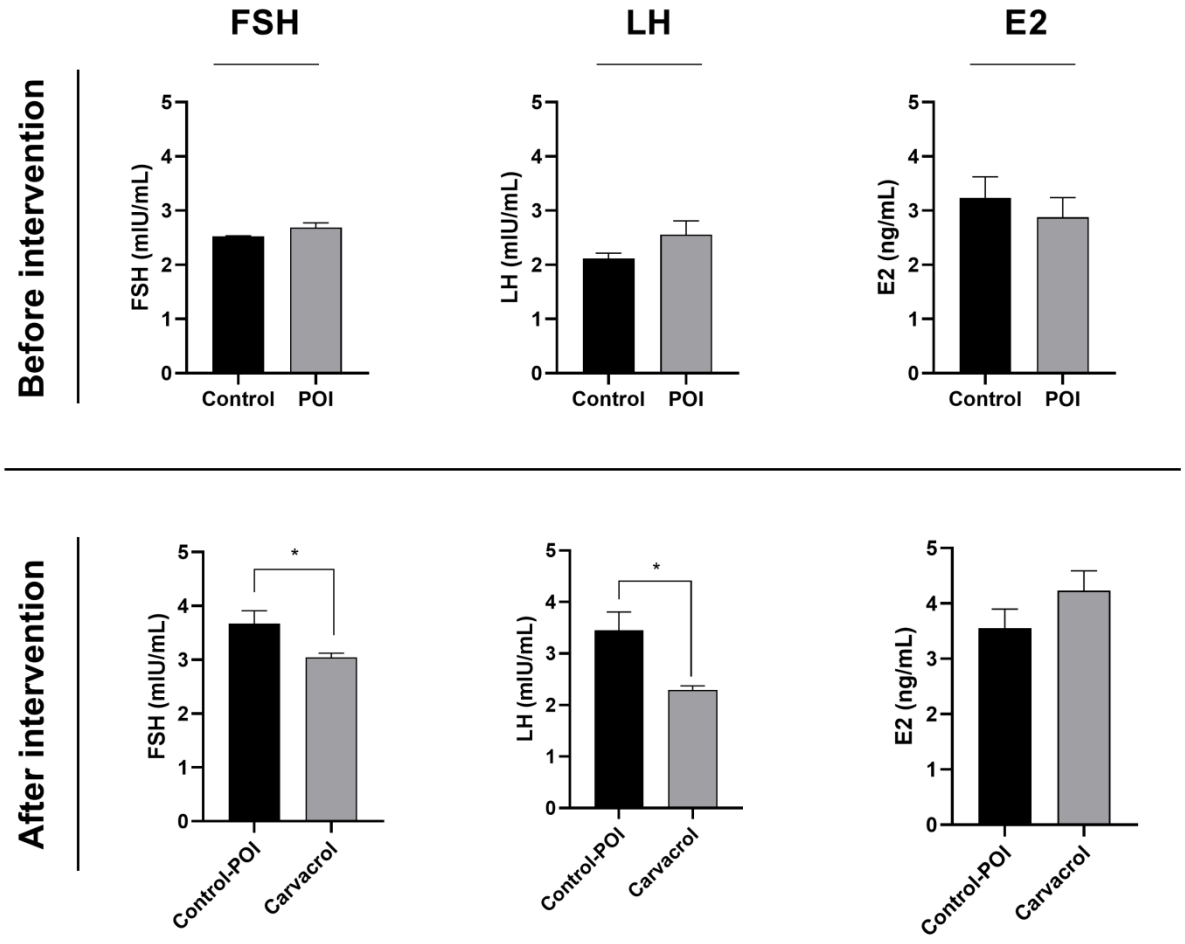

Figure 3. Serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2), before and after Carvacrol administration. *P < 0.05 (n = 3).

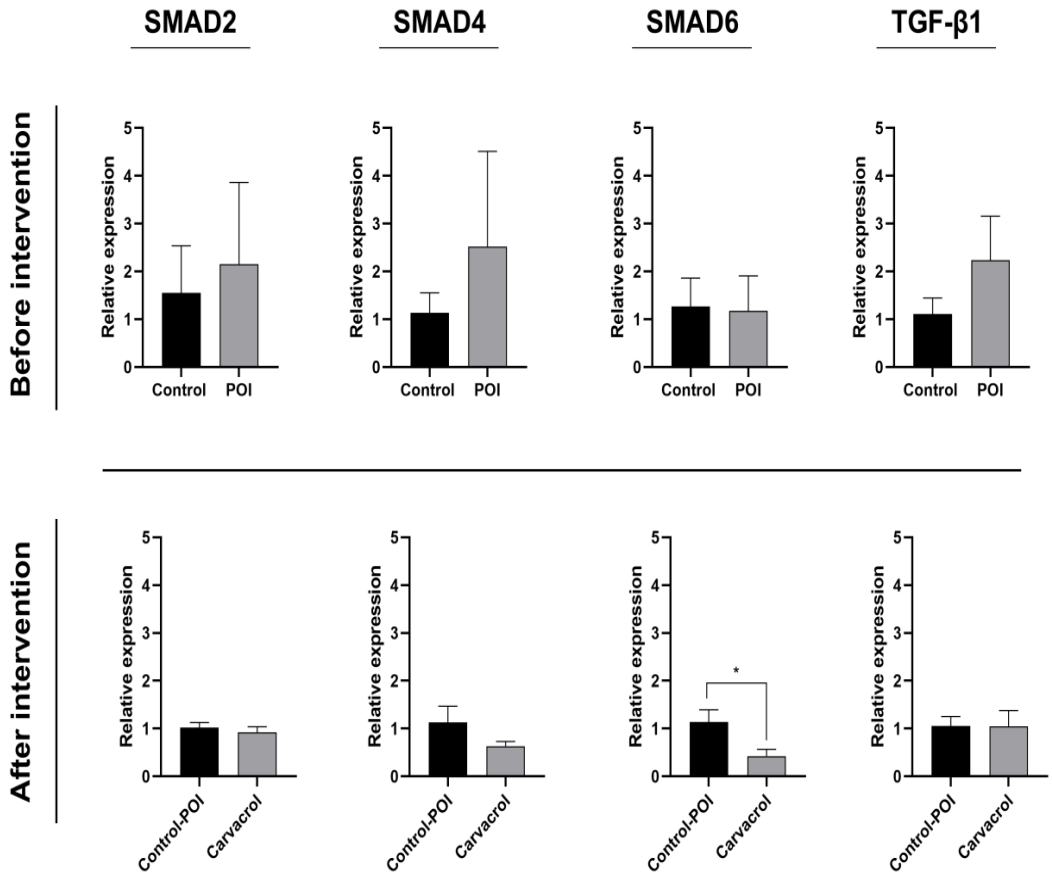

Figure 4. Relative expression of Smad2, Smad4, Smad6 and TGF-β1 genes before and after Carvacrol administration. *P < 0.05 (n = 3).

LH levels. Additionally, E2 content is normally high and decreases after CTX administration. Our study found that intervention with Carvacrol significantly decreased FSH and LH and increased E2 in Carvacrol-treated rats. Thus, Carvacrol can alter the serum level of sex-related hormones in POI rats. Using stem cells, Guihai and colleagues also showed that the levels of FSH, LH, and E2 hormones can be regulated by using CTX in POI model (30). Folliculogenesis is an important aspect of ovarian function and reproductive biology (31). In POI patients, healthy follicles are reduced due to atresia and subsequent fibrosis. In this study, we observed an increase in all types of healthy follicles (primordial, primary, secondary, and antral) in POI rats after Carvacrol administration, accompanied by a decrease in atretic follicles. In line with previous data (Bahrehbar and Qin), an increase in the healthy follicle population following treatment was achieved (24, 32).

Carvacrol has been the subject of significant research in recent years, exploring its biological effects in various clinical applications. Several studies have demonstrated that Carvacrol possesses multiple biological properties (14, 33, 34).

Carvacrol can suppress pro-inflammatory cytokines and oxidative stress response in ovarian tissue, thereby improving ovarian function (15). Here, we monitored Smad genes in ovarian tissues treated with Carvacrol. The TGF-β/Smads pathway is known to modulate and suppress inflammatory factors, with active TGF-β signaling predominantly mediated by the Smad pathway (35). Dysregulation of this pathway is implicated in reproductive disorders, including POI, PCOS, and tissue fibrosis (21, 36, 37). The expression of Smad2 and 4 genes is increased prior to treatment and decreased following Carvacrol intervention. Conversely, Tgf-β1 expression showed the opposite pattern. Overall, these findings suggest that Carvacrol modulates the expression of Smad pathway genes, highlighting the significance of this study. Notably, Chetan et al. also reported similar results, showing that Carvacrol increased fibrotic protein levels through the TGF-β1/Smad signaling pathway, thereby reducing oxidative stress, inflammation, and kidney fibrosis (17). These findings could potentially provide a novel approach for diagnosing POI by evaluating Smad gene expression. Furthermore, Carvacrol may be a viable treatment option in clinical settings, in conjunction with tuberculosis therapy.

Conclusion

Biochemical and histopathological studies have confirmed that animals exposed to CTX leads to ovarian inflammatory response, tissue fibrosis, and infertility. In our study, Carvacrol significantly reduced atretic follicles. Carvacrol also normalized the levels of sex hormones and the expression of Smad pathway genes. Based on our data, Carvacrol not only blunts the detrimental effects of CTX on ovaries but also preserves ovarian follicles and improves their function. Data suggest that Carvacrol can be used for controlling ovarian toxicity and infertility in animals. However, several basic and clinical studies are needed to investigate the TGF-β/Smads pathway and its relationship with tissue fibrosis.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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