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Original Article

Systems Pharmacology Uncovers Multiple Mechanisms of Erxian Decoction (二仙汤) for Treatment of Premature Ovarian Failure*

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ABSTRACT **Objective:** To predict the chemical compositions and drug targets and to systematically dissect the pharmacological mechanism of Erxian Decoction (二仙汤, EXD) as a treatment for premature ovarian failure (POF) using a systems pharmacology approach. **Methods:** The compounds present in EXD were obtained from three databases. The active ingredient was identified by analyzing the values of oral bioavailability (OB), drug-likeness (DL), and Lipinski's rule (LR). The active ingredients were further searched in research articles, drug targets in the DrugBank database, and the C-T and T-P networks, as well as by pathway analysis using the Cytoscape platform. **Results:** A total of 728 compounds were identified in EXD. Of these, 59 were identified as active compounds that conformed to the criteria with OB ⩾30% and DL ⩾0.18. By further searches in the literature, 126 related targets were identified that could interact with the active compounds. Additionally, it was found that the beneficial effects of EXD in POF are probably exerted via regulation of the immune system, modulation of estrogen levels, and anti-oxidative activities, and that it may act in a synergistic or cooperative manner with other therapeutic agents. **Conclusions:** The systems pharmacology approach is a comprehensive system that was used to elucidate the pharmacological mechanism of EXD as a treatment for POF. The results of this study will also facilitate the application of traditional medicine in modern treatment strategies.

KEYWORDS premature ovarian failure, Chinese medicine, Erxian Decoction, systems pharmacology, pharmacological mechanism

The end of a woman's reproductive ability, which often occurs due to ovarian reserve failure, is indicated by the arrival of menopause, which is thought to be the last menstrual period.[1] For most women, regardless of racial differences, menopause starts at the age of 47, varying on an average by 5–8 years.[2] However, aberrations during ovulation may result in premature exhaustion of the follicles leading to premature menopause, which indicates a complete decline of ovarian function in women. This phenomenon is termed as premature ovarian failure (POF), since it occurs before the age of 40, also known as primary ovarian insufficiency.[3] Women with POF suffer from hot flashes, vaginal dryness, and other menopausal symptoms, along with an increased risk of osteoporosis, which reduces the quality of life of the patients. The reasons for women with POF to have symptoms similar to natural menopause are also responsible for an early loss of fertility. The loss of fertility is attributed to several reasons, including accelerated follicular apoptosis, inability of the residual follicles to express ovulation signals, initial decrease in ovarian reserve at birth, which is related to immunity or any of the aforementioned factors.

Erxian Decoction (二仙汤, EXD) has been repeatedly screened and verified by Prof. ZHANG Bo-na for the treatment of perimenopausal syndrome (menopausal syndrome), and a prescription was eventually developed in the 1950s. This prescription warms the Shen (Kidney) yang, Shen essence, and Shen fire, regulating Chong and Ren. In Chinese

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medicine (CM), experts often use EXD for the treatment of POF,[4] but its specific mechanism of action is not clear.

CM is a complex multi-component, multi-targeting system comprising synergistically acting components. However, it is difficult to systematically explain the complex relationships of CM using a single theory. The recent systems biology technique, systematic pharmacology, is an extension of classical pharmacology, and provides a new approach to explore CMs across multiple complexity scales, ranging from molecular to cellular, tissue and organismic levels.[5] Systematic pharmacology has made a significant contribution to the study of CMs from the evaluation of drug action [absorption, distribution, metabolism, excretionor (ADME) properties] to target prediction and network/pathway analyses.[6] This study applied the theory of systems pharmacology to explain the molecular mechanism of EXD for the treatment of POF, which can act as a reference for future experimental research including pharmacodynamic component analysis and understanding the mechanism of EXD activity.

METHODS

Database of Active Compounds

EXD comprises of *Curculiginis Rhizome* (C.R.), *Epimrdii Herba* (E.H.), *Morindae Officinalis Radix* (M.O.R.), *Phellodendri Chinrnsis Cortex* (P.C.C.), *Angelicae Sinensis Radix* (A.S.R.), and *Anemarrhenae Rhizoma* (A.R.). The chemicals present in the 6 herbs that comprise EXD were collected by manually searching databases, including, the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP, http://lsp.nwsuaf.edu.cn/tcmsp.php),[7] the Database of Chinese Academy of Sciences (http://www.organchem.csdb.cn/scdb/default.htm) and the CM Database of Taiwan, China (http://tcm.cmu.edu.tw/join-us.php). In order to screen out the potential active compounds from these herbs, we applied a filtering method which incorporated oral bioavailability (OB), drug-likeness (DL) and the Lipinski's rule (LR).

OB, one of the most important pharmacokinetic parameters among all medicinal properties (ADME properties), refers to the relative amount and the rate at which a given compound can be absorbed into the systemic circulation by oral administration.[8] OB is usually one of the key indices of bioactive molecules having drug-like activities. OBioavail 1.1 is a computationally-built mathematical model developed by Prof. WANG Yong-hua's laboratory in Northwest A&F University.[9] It uses multiple linear regression (MLR), partial least squares (PLS), and support vector machines (SVM) for model construction, and calculates the OB of compounds. In this study, we set the OB at ⩾30% as a screening index.

DL refers to the similarity between compounds and all known drugs in the DrugBank database (https://www.drugbank.ca/). We screened compounds that were not suitable for chemicals in terms of the DL, and excluded these compounds prior to target prediction. In this study, the DL was obtained by calculating the Tanimoto coefficient as follows,

$$f(A, B)= \frac{A \cdot B}{|A|^2+|B|^2-A \cdot B}$$

Where A stands for the new compound, B represents the average class of all the 6,511 compounds in the DrugBank database. The calculation was carried out by the Dragon software, using SVM. A value of DL ⩾0.18 (the average of the whole similarity) means that there is a certain similarity between the tested compound and the compounds of the DrugBank database. By using this method, we were able to screen all the compounds having medicinal properties, for carrying out further research.

LR is a basic law put forward by Christopher A. Lipinski, a senior medicinal chemist at Pfizer Inc. for screening molecules in 1997. Compounds which meet the criteria of LR have better pharmacokinetic properties and a higher bioavailability during metabolism in organisms, and are therefore more likely to be ingested as oral drugs. The screening criteria are: (1) the molecular weight of the compound should be less than 500; (2) the number of hydrogen bond donors should be less than 5; (3) the number of hydrogen bond receptors should be less than 10; (4) the ratio of the distribution coefficient of lipid to water should be less than 5. Only compounds that meet at least 3 of these conditions are considered to be active oral drugs.[9] In this work, all of the active compounds screened should compliance (1), (2) and (3) and some of them met the 4th condition.

Considering that some of the major active compounds may not meet the screening criteria

above, and that some of the existing compounds may not be included in these three databases, we further look for active compounds in the literature.

Drug Targeting

Drug molecules by combining with a specific protein or nucleic acid target, adjusts the biological activity of the protein or nucleic acid, to exert a therapeutic effect. In order to understand the mechanism of action of CM prescriptions, the active molecular ingredient of the CM prescription must be firstly identified. In order to understand disease pathogenesis and the pharmacological mechanism of EXD, we need to construct a compound-target (C-T) network. In this work, guided by a large number of drug-target relationships, we employed the weighted ensemble similarity (WES) algorithm for predicting the direct targets of the active ingredient.[10] The sensitivity of the WES model was 85%, the specificity was 71%, the accuracy was 78%, and the area under the curve (AUC) was 0.85. In this work, according to the possibilities of the C-T interactions in the WES model, the targets with a likelihood score ⩾7 were selected as candidate targets for further analyses. In addition, the candidate targets were further mapped to Uniprot (http://www.uniprot.org/) for standardization.

Construction of Compound-Target-Pathway Network

The construction of the compound-target-pathway network can help identify the potential targets for each compound, understand the mechanism of drug treatment at a macroscopic level, and discover the new roles of CM prescriptions. For a clear understanding of the mechanisms of EXD activity in POF at a network level, we constructed two kinds of visualized networks in this study: C-T network and target-pathway network (T-P network). The networks were constructed with Cytoscape version 3.2, which is an open source, free software for network visualization and analyses, and combines basic data into a visual network for network parameter analyses and biometric annotations. We used the Network Analyzer plugin of Cytoscape 3.2 to analyze the two important topological parameters of network nodes, degree and betweenness. We utilized the targets as baits to fish out corresponding pathways from the KEGG database (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/).

POF Pathway Analysis

Based on the current situation of clinical treatments for POF, and the available research reports of basic experiments on the pathogenesis of POF, we constructed an integrated "POF pathway", which was manually assembled after excluding the pathways not directly or closely related to POF in the T-P network.

RESULTS

Construction of Compounds Database from EXD

From research literature surveys and the aforementioned three databases, 728 compounds were identified in EXD, including 78 compounds from C.R., 130 compounds from E.H., 174 compounds from M.O.R., 140 compounds from P.C.C., 125 compounds from A.S.R., and 81 compounds from A.R..

Screening Active Compounds

We proceeded to screen the 59 active compounds of EXD. Sifting through the 59 (8 from C.R., 19 from E.H., 15 from M.O.R., 10 from P.C.C., 9 from A.R., 3 from A.S.R.) active compounds (Appendix 1), we found that 52 of them met the following criteria: OB ⩾30% and DL ⩾0.18. Beta-sitosterol (M04) is a common component of C.R., M.O.R., P.C.C., and A.S.R., with high OB (36.91%) and DL (0.75) values. Sitosterol (M05, OB=36.91%, DL=0.75) is a component common to E.H. and M.O.R. Stigmasterol (M08, OB=48.83%, DL=0.76) is a component common to C.R., A.R., P.C.C., and A.S.R., and has anti-inflammatory, anti-oxidative, anti-tumor and other pharmacological activities. Anhydroicaritin (M34, OB=45.41%, DL=0.44) and 3,5,7-trihydroxy-4'-8-prenylflavone-3-o-rhamnopyranoside (baohuoside-1, M40, OB=3.7%, DL=0.84) are components common to both E.H. and A.R.. The additional 7 compounds were obtained through literature mining, 2,6-dimethoxybenzoic acid (M32, OB=62.68, DL=0.05), curculigoside (M31, OB=14.89%, DL=0.71), 3,5,7-trihydroxy-4'-8-prenylflavone-3-o-rhamnopyranoside (baohuoside-1, M40, OB=3.7, DL=0.84), ferulic acid (M06, OB=54.97, DL=0.06), mangiferin (M44, OB=13.74%, DL=0.75), phellodendrine (M25, OB=2.5, DL=0.58) and linoleic acid (M03, OB=41.90%, DL=0.14), which were also included for further studies.

Drug Targeting

We obtained 126 candidate targets (Appendix 2) of the 59 active compounds, with 734 connections

between them. The results showed that most of the compounds have more than one target, demonstrating various pharmacological effects of the bioactive molecules. For instance, quercetin (M02) from E.H. can interact with 55 targets, and beta-sitosterol (M04) from C.R. targets 38 different proteins, which embodies the "multi-target" concept of CMs. In order to validate whether the 126 selected targets indeed correlate to POF, we performed a Gene Ontology (GO) analysis for their biological processes. The 86 potential targets (degree ⩾3) were mapped with the bioinformatics resources of DAVID (The Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov) by systematically analyzing their biological process. The top 15 significantly enriched GO terms are enlisted in Figure 1.

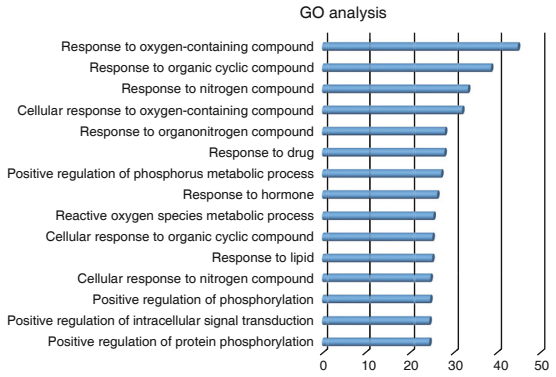

Figure 1. GO Analysis of Therapy Target Genes of EXD

Notes: The y-axis shows significantly enriched 'Biological Process' categories in GO of the target genes, and the *x*-axis shows the enrichment scores of these terms ($-\log_{10} P$ value. The *P* value means the significance of these genes being enriched in the GO term)

CT Network

A graph of C-T interactions was built based on 184 nodes (59 potential compounds and 125 potential targets) and 995 edges. C-T network analyses displayed that the average degree of the number of targets per compound is 5.825, elucidating the multi-target properties of EXD (Figure 2).

TP Network

Based on the results of C-T network analyses, we got 86 (degree ⩾3) targets for further research. The results displayed that the 86 targets further mapped to 114 pathways. Meanwhile, numerous pathways (Appendix 3), 63 out of 114, were also regulated by multiple target proteins (degree ⩾6), which might be the key factors for POF. The T-P network (Figure 3) showed an average degree of 9.22 per target and 7.17 per pathway, while 5 of the 86

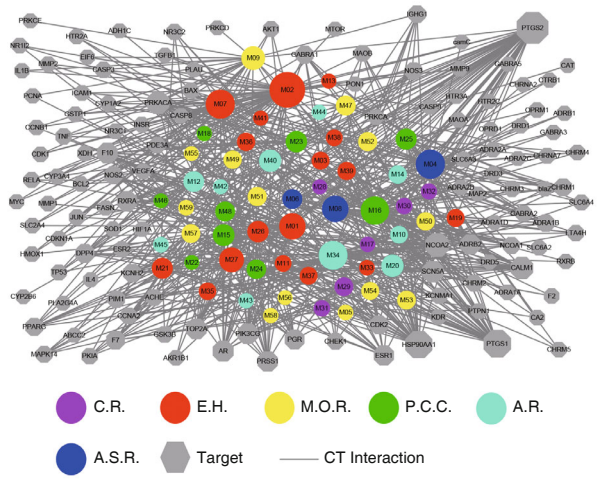

Figure 2. C-T Network Analysis of EXD

Notes: A compound node and a protein node are linked if the protein is targeted by the corresponding compound. Node size is proportional to its degree.

targets did not map into the network. We discovered that several target proteins (32 out of 81) mapped to multiple pathways (degree ⩾6).

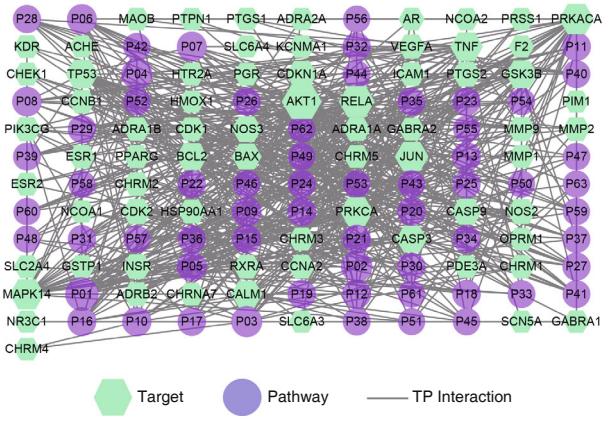

Figure 3. T-P Network Analysis of EXD

Notes: The T-P network is built by a target and a pathway if the pathway is lighted at the target. Node size is proportional to its degree. The information of pathways is obtained by mapping the target proteins to the KEGG pathway database.

POF Pathway

An integrated "POF pathway" was constructed by integrating the key pathways that were obtained through the T-P network analyses, including the PI3K-Akt signaling pathway (map04151, degree=18), the estrogen signaling pathway (map04915, degree=11), the interleukin (IL)-17 signaling pathway (map04657, degree=11), the cAMP signaling pathway (map04024, degree=9), the apoptosis pathway (map04210, degree=9), the vascular endothelial growth factore (VEGF) signaling pathway (map04370, degree=8) and the GnRH signaling pathway (map04912, degree=6).

The target proteins of the integrated "POF pathway" exhibit markedly close functional relationships with the proteins associated with POF. The POF pathway can be demarcated into three representative therapeutic modules, the immunoregulation module, the estrogen module and the oxidation-resistant module (Figure 4).

DISCUSSION

In this work, 59 active compounds were identifed in EXD. Beta-sitosterol is a common component of C.R., M.O.R., P.C.C., and A.S.R., and experimental studies confirm that it can improve osteoporosis and has anti-oxidative properties.[12] Epimedium glycoside is one of the main components of the flavonoids in E.H., has a very strong biological activity, with significant effects on a variety of organs, especially the endocrine and the antitumor immune system.[14,15] It can substantially inhibit mitochondrial swelling, reduce malonaldehyde (MDA) content, and increase the superoxide dismutase (SOD) activity, which has obvious roles in promoting follicular activity in rats having populations of cavity follicles.[14]

Mangiferin was confirmed as the main component of A.R. with effects of antioxidant, immune regulation, antiviral, and antitumor.[16] The pharmacopoeia published in 2015 reports that ferulic acid is the main active principle of A.S.R..[17] Experimental studies showed that it has anti-oxidative, free radical scavenging and cell-protective effects.[18] Quercetin exhibited the highest degree of the number of interactions with various protein targets. Studies have shown that quercetin inhibits uterine tumor cell proliferation,[19] by significantly raising caspase-3 expression levels, inhibiting HeLa cell proliferation and inducing their apoptosis, thus playing a role in cervical cancer inhibition and promoting the development of the mature follicle. Kaempferol (M07) can effectively reduce harmful immune responses such as chronic inflammation and autoimmunity.[20] We speculated that the aforementioned ingredients might be the crucial elements in the treatment of POF.

The results indicated that one target can be simultaneously targeted by multiple compounds from different herbs, which might contribute to the synergistic or additive effects of EXD in the treatment of POF. For instance, PTGS2 has the highest degree up to 45, which means that it is targeted by 45 active compounds

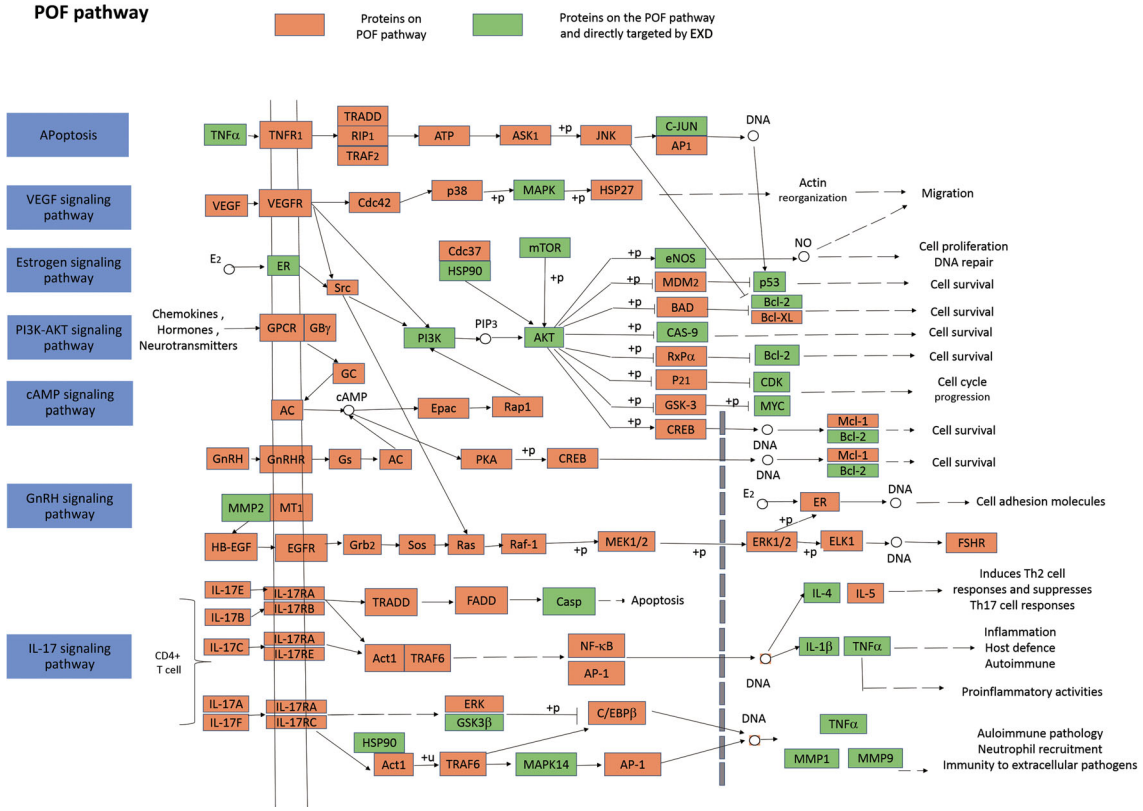

Figure 4. Distribution of Target Proteins of EXD on Compressed 'POF Pathway'

Notes: Seven pathways (lightsky-blue) form the compressed POF pathway. Arrows represent activation effect, T-arrows represent inhibition effect and segments show activation effect or inhibition effect.

identified from EXD, which might be associated with inflammation.[21] PTGS1 (degree=34) may promote cell proliferation during tumor progression, and alternative splicing results in multiple transcript variants. HSP90AA1 (degree=33) is a molecular chaperone which functions as a dimer and aids protein folding and quality control for a large number of 'client' proteins, and has intrinsic ATPase activity. All of these results indicate that drug molecules have synergistic effects and synergize for the treatment of the disease.

As we know, there are at least three major hypotheses for the pathogenesis of POF, which are not exclusive of each other, including autoimmunity, hypoestrogenism and oxidative damage.[22,23] Therefore, targets which are involved in the pathologies of POF and the aforementioned autoimmune diseases are potential therapeutic targets for POF. The GO analysis show that the majority of these targets are strongly associated with various biological processes, including the regulation of autoimmunity (positive regulation of phosphorus metabolic process), hypoestrogenism (response to hormone), and anti-oxidative damage (response to oxygen-containing compound, cellular response to oxygen-containing compounds and reactive oxygen species metabolism).

These pathways strongly interact with targets involved in different crucial pathways. The IL-17 signaling pathway (map04657, degree=11) has autoimmune effects by interactions with corresponding receptors which activate downstream pathways. Some targets involved in resisting apoptosis and oxidative damage are located in the apoptosis pathway (map04210, degree=9) and the PI3K-Akt signaling pathway (map04151, degree=18). GnRH agonists and estrogen are often used to treat POF, which are associated with the estrogen signaling pathway (map04915, degree=11) and the GnRH signaling pathway (map04912, degree=6).[24] In addition, some pathways like the VEGF signaling pathway (map04370, degree=8), the PI3K-Akt signaling pathway, the cAMP signaling pathway (map04024, degree=9), have been testified as accurate target pathways associated with POF treatment.[25-27] These illustrate that EXD integrates multiple signaling pathways to regulate the immune system, modulate estrogen levels and regulate anti-oxidative damage.

The activation of humoral and cell-mediated immune mechanism is likely to be involved in the ovarian destruction seen in POF. For instance, TNFα in the IL-17 signaling pathway plays a vital role in inflammation and autoimmunity by inducing the release of $CD4^+$ human peripheral blood T lymphocytes. Baohuoside-1 (M40) from E.H. might play a vital role in the treatment of POF by regulating TNFα. In addition, luteolin (M01) treatment causes a dose-dependent and statistically significant decrease in VEGF secretion, via its effect on pro-inflammatory cytokines.[28] At the same time, luteolin significantly inhibits the production of IL-1β and tumor nerosis factor (TNF)-α,[28] which are important biological mediators in inflammation and immunity in the IL-17 signaling pathway. All these indicate that EXD may cure POF by regulating the immune system.

It is well known that POF is characterized by hypoestrogenism and high levels of follicular stimulating hormone (FSH). For instance, the MMP2 in the GnRH signaling pathway which is significantly down-regulated in rats with POF after treatment with genistein,[27] is modulated by luteolin, quercetin from E.H., and rutaecarpine (M23) from P.C.C., which can significantly reduce MMP2 expression in cancer cells.[26,28] The inhibition of MMP2 can ultimately reduce FSHR expression. FSH binds selectively to FSHR on the surface of granulosa cells (GCs), whose activation depends on cAMP signaling pathways and GnRH signaling pathways.[26] FSH activates the adenylyl cyclase through the cAMP signal transduction pathway, which increases the cAMP levels and activates PKA. The downstream target gene P450 is subsequently regulated, which activates the P450 aromatase for converting testosterone into E_2. The active ingredients of C.R. and E.H. identified in this study, can significantly increase cAMP levels. These show that EXD may cure POF by increasing estrogen.[31] Some targets in the PI3K-Akt signaling pathway engage in creating equilibrium between cell survival and apoptosis.

The process of apoptosis of GCs is induced by oxidative stress, which plays an important role in the pathogenesis of POF.[30] The PI3K-Akt signaling pathway also takes part in the regulation of oxidative stress by inhibiting the apoptosis of GCs. Some in targets in the PI3K-Akt signaling pathway engage creating equilibrium between the levels of cell survival and apoptosis. For instance, diosgenin (M12) from

A.R. down-regulates the levels of mTOR in cells in a dose- and time-dependent manner.[33] The inhibition of mTOR can lead to low-expression of Akt. Lower expression levels of Akt increases the expression of MDM2 which in turn inhibits p53 expression, thus up-regulating the expression of BAD, which reduces the expression of bcl-2, and depresses caspase-9 expression, and inhibits cells survival. Kaempferol from E.H. significantly decreases pro-inflammatory cytokines and TNF α in the apoptosis pathway.[34] At the same time kaempferol exhibits its anti-oxidant activity by inhibiting MDA expression and promoting SOD activities in the heart, lungs, and liver. The preceding information suggests that EXD may treat POF by regulating oxidation.

Conflicts of Interest

The authors declare that they have no competing interests.

Author Contributions

Du B, Liu LH and Ai H developed the concept of the study. All authors contributed to data accumulation. Du B, Liu LH and Lv YJ contributed to data analysis. All authors contributed to data interpretation. Du B and Ai H wrote the manuscript. All authors contributed to revisions of the manuscript, and approved of the final submission.

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