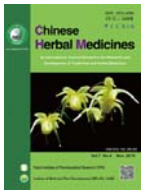

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## Original article

# Investigation of Mechanism of Premature Ovarian Failure Regulation by Kidney-tonifying Herbs and Liver-clearing Herbs in Dingjing Decoction

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ABSTRACT

**Objective** To investigate the mechanisms through which kidney-tonifying herbs (KTHs) and liver-clearing herbs (LCHs) in Dingjing Decoction (DJD) regulate premature ovarian failure (POF). **Methods** One hundred and fifty Sprague-Dawley rats were randomly divided into five groups such as control, model, KTHs, LCHs, and DJD groups. POF-related biological molecules were examined. Factor analysis was performed to investigate the regulatory networks and key biomolecules involved in mediating POF after treatment with KTHs and LCHs. **Results** The master regulatory factors in the reproductive endocrine network associated with KTHs intervention included four molecules in the pituitary-ovarian axis, cortisol (CORT) in the target gland of pituitary-adrenal axis, and some molecules in the hypothalamus. In contrast, the master regulatory factors associated with LCHs intervention included four molecules in the pituitary-ovarian axis and some molecules in the hypothalamus; No biomolecules in the pituitary-adrenal axis were involved in the LCH-mediated mechanisms. Gonadotropin-releasing hormone (GnRH), which was identified as a common biological molecule in the hypothalamus, was involved in regulating the reproductive endocrine network in association with KTHs intervention. **Conclusion** KTHs directly regulates biological molecules in the pituitary-adrenal axis and indirectly regulates those in the pituitary-adrenal axis through the hypothalamus, while the LCHs only exert its effects indirectly. GnRH is the key biological molecule associated with KTHs intervention.

*Key words*

corticotropin-releasing hormone; Dingjing Decoction; factor analysis; kidney-tonifying herbs; liver-clearing herbs; signaling mechanism; premature ovarian failure; pituitary-adrenal axis



## 1. Introduction

Premature ovarian failure (POF), a common gynecological endocrine disease, is characterized by amenorrhea, dysgenesis, hypoestrogenism, and hyper-gonadotropinism resulting from exhaustion of ovarian follicles before the age of 40 (Robert,

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2009). Premature menopause in women of child-bearing age may result in a heavy psychological burden and influence the quality of women's marriages, leading to a series of psychological and social problems. In Western medicine, treatment with estrogen is a commonly used method for preventing POF, however, long-term therapy may induce gynecological tumorigenesis.

Science of traditional Chinese medicine (TCM) focuses on tonifying the kidneys and soothing the liver when treating POF induced by kidney deficiency and liver stagnation. The Dingjing Decoction (DJD), which is described in *Fu Qingzhu's Gynecology*, written by Fu Shan during the early Qing dynasty period in China, showed significant beneficial effects in the treatment of abnormal menstruation induced by kidney deficiency combined with liver stagnation, including irregular menstruation, amenorrhea, oligotrichia, and dysgenesia (Gong, 2012). DJD consists of *Angelicae Sinensis Radix* 30 g, *Paeoniae Alba Radix* 30 g, *Semen Cuscutae* 30 g, *Rehmanniae Radix Preparata* 15 g, *Dioscoreae Rhizoma* 15 g, *Poria* 9 g, *Bupleuri Radix* 1.5 g, and *Schizonepetae Herba* 6 g. Among them, *Cuscutae Semen* and *Rehmanniae Radix Preparata* can tonify the kidney and replenish the essence; *Angelicae Sinensis Radix* and *Paeoniae Alba Radix* can nourish the liver; *Dioscoreae Rhizoma* and *Poria* can strengthen the spleen and replenish the *qi*; *Bupleuri Radix* and *Schizonepeta tenuifolia* can nourish the liver and resolve depression. Although there are only eight herbs in the formula, the prescription is quite specific and well formulated and is highly efficacious at nourishing the kidney and soothing the liver. This is a classical formula for treating POF induced by kidney deficiency combined with liver stagnation.

Currently, studies on the mechanisms of DJD are limited to the identification of only a few POF-related biological molecules, and the results can not fully illustrate the mechanisms through which DJD exerts its beneficial effects. According to TCM science, the body is considered as a complicated organized system. In order to shed light on mechanisms of DJD activity, we carried out an extensive literature search to systematically investigate POF-related biological molecules. Multiple studies have demonstrated that the following biological molecules are involved in mediating POF (Mitchell et al, 2005; Kawauchi and Sower, 2006; Zheng et al, 2009; Zeng et al, 2007; Kadekaro, 2004; Dufourny and Skinner, 2002; Tong et al, 2005; Raga et al, 2008; Waschul et al, 2003; Ott et al, 2014; Rah et al, 2013; Yorgun et al, 2013): β-endorphin (β-EP), interleukin-1 (IL-1), nitric oxide synthase (NOS), nitric oxide (NO), estrogen receptor (ER), progesterone receptor (PR), gonadotropin-releasing hormone (GnRH), and corticotropin-releasing hormone (CRH) in the hypothalamus; follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol ($E_2$), and progesterone (P) in the pituitary-ovarian axis; and adrenocorticotropic hormone (ACTH) and cortisol (CORT) in the pituitary-adrenal axis.

The factor analysis method, which has been effective for multiple factor correlation analysis in the processing of animal experiment data, may help to elucidate the networks through which these molecules are involved in mediating POF in response to DJD treatment. In our previous studies, we used factor analysis to reach preliminary conclusions concerning the mechanisms through which DJD (as a whole) prevents POF (Zhan et al, 2013). To further describe the different mechanisms of kidney-tonifying herbs (KTHs) and liver-clearing herbs (LCHs) in this formula, the components of DJD were divided into KTHs and LCHs according to their efficacy in the present study; KTHs consist of *Cuscutae Semen* 30 g and *Rehmanniae Radix Preparata* 15 g, while LCHs consist of *Bupleuri Radix* 1.5 g and *Schizonepetae Herba* 6 g. POF-related biological molecules were selected for factor analysis of the two formulas. The correlations among biological molecules of the two groups and efficacy in POF treatment were investigated by factor analysis, and potential interactions among the biological molecules were evaluated. Our results provided the insights into the mechanisms of KTHs and LCHs in the treatment of POF using DJD and the key targets of these two formulas.

## 2. Materials and methods

### *2.1 Animals*

Female specific pathogen-free (SPF)-grade Sprague-Dawley (SD) rats, weighing 200−220 g, as well as the standard basal diet, were supplied by Guangdong Medical Laboratory Animal Center. The animal license number was SCXK 2008-0002. The rats were acclimatized to the facilities for 3 d, and the estrum cycle was observed every day. Rats with two continuous estrum were chosen for experiments and randomly divided into five groups ($n$ = 30): control, model, DJD, KTHs, and LCHs groups.

### *2.2 Plant materials, reagents, and equipment*

The medical materials of *Angelice Sinensis Radix*, *Paeonia lactiflora Radix*, *Semen Cuscutae*, *Rehmannia Glutinosa Radix*, *Dioscoreae Rhizoma*, *Poria*, *Bupleuri Radix*, and *Schizonepetae Herba* were all purchased from Medical Materials Company of Guangzhou City, and authenticated by Prof. Shu-yuan Li from Guangdong Pharmaceutical University. The voucher specimens were deposited in our laboratory of School of Traditional Chinese Medicine, Guangdong Pharmaceutical University. The 0.9% sodium chloride injection was purchased from Henan Tailong Pharmaceutical Co., Ltd. (Batch No. 11103015). The TP kit was supplied by BioSino Bio-Technology and Science Inc. The NOS kit was obtained from Nanjing Jiancheng Bioengineering Institute. CRH, GnRH, β-EP, IL-1, and NO kits were provided by Beijing Sino-uK Institute of Biological Technology. $E_2$ and P kits were purchased from Sigma Corporation. The Chemistry Analyzer 7160 was provided by Hitachi (Japan), and the TDL80-2B centrifuge was purchased from Anting Scientific Instrumental Factory (Shanghai, China). The R-911 RIA Counter was obtained from the University of Science and Technology of China. The acousto-optical-electric stimulator was made in our laboratory.

### *2.3 Animal experimental protocol*

#### *2.3.1 Animal model preparation*

Acousto-optical-electric stimulation (acousto-optical for 10 s, acousto-optical-electric for 60 s, and electrical stimulation for 5 s) was applied to prepare the model for 20 d, five times per day, at random intervals (Wang et al, 2002). Each process was completed within 1 h. Acousto, optical, and electrical stimuli used the following constant parameters: 1) sound intensity of 65 dB; 2) illumination intensity of 500 lux with shining and frequency of 1/s, 500 lux with lights on, and 300 lux with lights off; and 3) voltage of 24−36 V.

#### *2.3.2 Administration*

Drugs were administered at the same day post-modeling. Distilled water was given to rats in the control and model groups, while drugs were given to corresponding experimental groups once per day for 25 d (drugs were given another continuous 5 d after stress stimulation for 20 d).

#### *2.3.3 Detection index*

Blood was collected from femoral artery and centrifuged at 3000 r/min for 20 min. Supernatants were transferred to a new tube. Hypothalami was separated, cleaned with filter paper, and then homogenized in 1 mL sodium chloride solution (containing 20 μL of 0.05 mol/L acetic acid) by grinding. The samples were centrifuged at 3000 r/min for 10 min, and supernatants were transferred to separate tubes. Precipitates were homogenized in 0.5 mL sodium chloride solution (containing 20 μL of 0.05 mol/L acetic acid) and centrifuged at 3000 r/min for 10 min. The supernatant was collected and combined with that obtained from the first centrifuge. Then 25 μL of 0.05 mol/L NaOH was added to adjust the pH value to about 7.4. The solution (10 mL) was used to determine the protein concentration. Radio-immunoassays were performed to detect $E_2$, P, LH, FSH, CORT, ACTH, and NO in serum and CRH, IL-1, β-EP, and GnRH in hypothalamus. NOS, PR, and ER in hypothalamus were examined by immunohistochemical methods.

### *2.4 Data preprocessing*

#### *2.4.1 Data normalization*

Indices obtained from various experimental biological molecules were normalized according to their different units and ranges.

#### *2.4.2 Elimination of exceptional values*

During the animal experiment and detection of biological molecules, multiple external and subjective conditions could result in unexpected changes, leading to the detection of exceptional values. In this study, the regulatory network of the biological molecules was identified by analyzing all the experimental groups, and significant differences among groups allowed data obtained from the control, model, DJD, KTHs, and LCHs groups to be classified into five different levels. Therefore, support vector machine classification-based calculations were performed to select the correctly classified samples for analysis and delete the incorrectly classified samples in order to realize the elimination of exceptional values.

### *2.5 Factor analysis*

Factor analysis is an important element of multivariate statistical analysis. It is often used to group the variables according to their correlations. After the grouping, the correlations of variables within groups were higher, while the correlations of variables between the groups were lower (Huang, 2004). These groups of variables, known as common factors, are hidden variables that usually can not be measured directly, but have practical significance. With proper interpretation and a solid background, common factors may provide reliable information. The process of factor analysis was as follows. First, data samples were normalized, and the correlation matrix R, its characteristic roots, and eigenvector were calculated. Secondly, the quantity of the principal divisors was fixed according to the accumulative variance contribution, and the component matrix was calculated to allow the simulation of the factor model. We used the factor analysis to study the correlations and interactions among the molecules in the two formula groups. Together with background knowledge and previous studies of POF, we attached the practical meanings to the common factors and revealed the biological mechanisms of KTHs and LCHs from DJD in the treatment of POF.

IBM SPSS Statistics software (ver. 20.0, USA) was used for factor analysis; principal component analysis was used for factor extraction, and the varimax procedure was used for factor rotation. The common factors were extracted based on the component matrix from orthogonal rotation. Scilab software (ver. 5.4.1, France) was used for the support vector classification during data preprocessing.

## 3. Results and discussion

### *3.1 Changes of weights of ovarian pituitary, ovary, adrenal glands, and changes of ovarian morphologic of rats with POF induced by psychological stress*

Our previous studies showed that the weights of ovarian pituitary, ovary, and adrenal glands of rats with POF induced by psychological stress were less significant, while ovarian tissue also showed a significant injury compared with normal rats (Wang et al, 2002), and it showed that our animal experimental protocol was successful.

### *3.2 Changes of contents of hormones on pituitary–gonad axis*

The contents of the hormones in rats of the model group on the pituitary-gonadal axis all had the significant difference compared with those in the normal group (Table 1), it also shows that our animal experimental protocol was successful. Meanwhile the contents of hormones in DJD, KTHs, and

LCHs groups on the pituitary-gonadal axis all have the significant difference compared with those in the model group (Table 1), indicating that DJD, KTHs, and LCHs all could adjust POF to varying degrees.

### *3.3 Factor analysis of POF-related biological molecules under intervention of KTHs*

In the KTHs intervention group, factor analysis was performed using the preprocessing values of POF-related biological molecules, including β-EP, IL-1, NOS, NO, ER, PR, GnRH, CRH, FSH, LH, $E_2$, P, ACTH, and CORT. Factor loading matrix was obtained after orthogonal rotation and the first five arrays were listed in Table 2.

The common factors in first five columns had a total variance contribution of 82.289%, indicating the extent to which these factors could be used to explain the observed effects. The higher the factor-loading value of a certain molecular was, the more it correlated with the common factor. The correlation was stratified into three levels based on

**Table 1 Changes of values of hormones on pituitary-gonadal axis ($\bar{x} \pm s$, $n$ = 30)**

| Groups | Doses /(mL·100g$^{-1}$) | FSH / (mIU·mL$^{-1}$) | LH / (mIU·mL$^{-1}$) | $E_2$ / (pg·mL$^{-1}$) | P / (ng·mL$^{-1}$) |
|---|---|---|---|---|---|
| normal | - | 10.94 ± 0.79 | 34.69 ± 2.04 | 20.84 ± 5.10 | 1.96 ± 0.55 |
| model | - | 11.73 ± 0.90$^{\triangle}$ | 31.42 ± 1.22$^{\triangle\triangle}$ | 16.00 ± 2.38$^{\triangle\triangle}$ | 1.46 ± 0.41$^{\triangle\triangle}$ |
| DJD | 1 | 9.85 ± 1.64$^{\triangle **}$ | 32.74 ± 1.19$^{\triangle\triangle **}$ | 20.14 ± 2.11$^{**}$ | 1.60 ± 0.29$^{\triangle}$ |
| KTHs | 1 | 10.39 ± 0.71$^{\triangle **}$ | 33.31 ± 1.26$^{\triangle **}$ | 18.93 ± 2.65$^{*}$ | 2.08 ± 0.76$^{**}$ |
| LCHs | 1 | 10.34 ± 0.51$^{\triangle **}$ | 32.25 ± 1.06$^{\triangle\triangle}$ | 19.37 ± 3.34$^{**}$ | 1.75 ± 0.31$^{*}$ |

Notes: $^{\triangle}P < 0.05$ $^{\triangle\triangle}P < 0.01$ *vs* normal group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model group.

**Table 2 Rotated component matrix of POF-related biological molecules following KTHs intervention (first five arrays)**

| Molecules | $f_1$ | $f_2$ | $f_3$ | $f_4$ | $f_5$ |
|---|---|---|---|---|---|
| β-EP | 0.232 | 0.876 | −0.200 | −0.093 | 0.020 |
| IL-1 | 0.397 | 0.806 | 0.247 | −0.043 | −0.105 |
| NOS | −0.895 | 0.090 | 0.189 | 0.001 | 0.198 |
| NO | −0.060 | −0.071 | 0.933 | −0.070 | 0.072 |
| ER | −0.046 | −0.039 | 0.071 | −0.062 | 0.961 |
| PR | 0.661 | 0.064 | −0.210 | −0.045 | −0.171 |
| GnRH | −0.404 | 0.699 | −0.147 | −0.444 | 0.088 |
| CRH | 0.419 | 0.791 | −0.079 | 0.211 | −0.023 |
| FSH | 0.853 | 0.445 | 0.071 | −0.010 | −0.048 |
| LH | 0.847 | 0.164 | 0.134 | −0.072 | 0.146 |
| E2 | 0.876 | 0.319 | 0.174 | −0.026 | −0.107 |
| P | −0.774 | −0.229 | 0.019 | −0.025 | −0.290 |
| ACTH | −0.150 | −0.030 | −0.074 | 0.914 | −0.052 |
| CORT | −0.591 | −0.261 | 0.318 | 0.237 | 0.015 |

The first eight molecules in the table were related to the hypothalamus, the four molecules in the middle were related to the pituitary-ovary axis, and the last two molecules were related to the pituitary-adrenal axis. The corresponding index of the underlined factor-loading values in each column belonged to the common factors. same as below

relevance. A factor-loading value below 0.4 indicated that there was little correlation between the molecule and the common factor; A factor-loading value over 0.6 suggested a strong correlation; And a factor-loading value between 0.4 and 0.6 indicated a possible correlation (Zhang et al, 2013). Accordingly, $f_1$was closely correlated with P, $E_2$, FSH, LH, NOS, and PR, and tended to have the correlations with CORT, GnRH, and CRH. $f_1$ had the highest variance contribution (42.658%), which suggested that $f_1$ was the common factor for master regulation of POF-related biological molecules associated with KTHs intervention. The common factor $f_1$ included four molecules in the pituitary-ovary axis, CORT in the target gland of the pituitary-adrenal axis, and some molecules in the hypothalamus, meanwhile compared the importance of five factors in all 14 indices, $f_1$ could be recognized as the master regulatory factor in the reproductive endocrine network associated with KTHs intervention. Our analysis also showed that $f_2$ was closely related to β-EP, IL-1, GnRH, and CRH and partly related to FSH, with a variance contribution of 15.154%; Thus, $f_2$ included molecules in the hypothalamus and pituitary-ovary axis, suggesting that $f_2$ was the regulator of the hypothalamus acting on pituitary-ovary axis during KTHs intervention. $f_3$ was only related to NO, with a variance contribution of 9.941%, indicating that NO had only a modest relationship with other molecules during KTHs intervention and that NO was relatively independent. $f_4$ showed a close relationship with ACTH and some relationship with GnRH, with a variance contribution of 7.809%, suggesting the affection between GnRH in the hypothalamus and ACTH in the pituitary-adrenal axis during KTHs intervention, so $f_4$ could be regarded as the regulator of the hypothalamus acting on pituitary-adrenal axis during

KTHs intervention. $f_5$ was only related to ER, indicating that ER had a modest relationship with other molecules during KTHs intervention and that ER was relative independent.

In addition, following treatment with KTHs, GnRH exhibited the close relationships with $f_1$, $f_2$, and $f_4$, indicating that GnRH was a hub of the master regulatory factor in the reproductive endocrine network and the regulators of the hypothalamus acting on the pituitary-ovary axis and the pituitary-adrenal axis. GnRH, CRH, and FSH, the common molecules of $f_1$ and $f_2$, were the hubs of the master regulatory factors in the reproductive endocrine network and the regulator of the hypothalamus acting on the pituitary-ovary axis.

### *3.4 Factor analysis of POF-related biological molecules under intervention of LCHs*

In the LCHs intervention group, factor analysis was performed using the preprocessing values of POF-related biological molecules, including β-EP, IL-1, NOS, NO, ER, PR, GnRH, CRH, FSH, LH, E2, P, ACTH, and CORT. Factor loading matrix was obtained after orthogonal rotation and the first five arrays were listed in Table 3.

**Table 3 Rotated component matrix of POF-related biological molecules following LCHs intervention (first five arrays)**

| Molecules | $f_1$ | $f_2$ | $f_3$ | $f_4$ | $f_5$ |
|---|---|---|---|---|---|
| β-EP | 0.554 | 0.754 | −0.141 | −0.051 | 0.012 |
| IL-1 | −0.039 | 0.006 | −0.037 | 0.035 | 0.964 |
| NOS | −0.632 | 0.365 | 0.600 | 0.117 | −0.123 |
| NO | 0.825 | 0.105 | 0.126 | 0.049 | 0.136 |
| ER | 0.305 | −0.041 | 0.320 | −0.679 | −0.330 |
| PR | 0.330 | −0.023 | −0.020 | 0.678 | −0.073 |
| GnRH | 0.051 | 0.941 | 0.045 | −0.064 | −0.051 |
| CRH | 0.556 | 0.703 | −0.209 | 0.072 | 0.155 |
| FSH | 0.850 | 0.199 | −0.331 | −0.013 | -0.052 |
| LH | 0.894 | 0.094 | −0.263 | −0.049 | −0.077 |
| E2 | 0.872 | 0.276 | −0.220 | 0.053 | −0.106 |
| P | −0.750 | −0.293 | 0.115 | −0.056 | 0.146 |
| ACTH | −0.175 | −0.098 | 0.857 | −0.256 | 0.001 |
| CORT | −0.292 | −0.241 | 0.523 | 0.480 | −0.090 |

The common factors in first five columns had a total variance contribution of 81.695%, indicating the extent to which these factors could be used to explain the observed effects. $f_1$ closely correlated with P, $E_2$, FSH, LH, NO, and NOS, and tended to have correlations with β-EP and CRH. $f_1$ had the highest variance contribution (43.476%), which suggested that $f_1$ was the common factor for master regulation of POF-related biological molecules associated with LCHs intervention. The common factor $f_1$ included four molecules in pituitary-ovary axis and some molecules in the hypothalamus, meanwhile compared the importance of five factors in all 14 indices, $f_1$ could be recognized as the master regulatory factor in the reproductive endocrine network associated with LCHs intervention. Our analysis also showed that $f_2$ was closely related to GnRH, β-EP, and CRH, with the variance contribution of 13.054%, only including molecules in hypothalamus, suggesting that $f_2$ was the regulator of hypothalamus itself during LCHs intervention. $f_3$ closely correlated with ACTH and NOS, and tended to have the correlations with CORT, with the variance contribution of 10.872%. $f_3$ included NOS and two molecules in pituitary-adrenal axis, suggesting that $f_3$ was the regulator of hypothalamus acting on pituitary-adrenal axis during LCHs intervention. $f_4$ showed the close relationship with ER and PR, some relation with CORT with the variance contribution of 7.873%, suggesting that $f_4$ was another regulator of hypothalamus acting on pituitary-adrenal axis during LCHs intervention. $f_5$ was only related to IL-1, indicating that IL-1 had a modest relationship with other molecules during LCHs intervention and that IL-1 was relative independent.

In addition, following treatment with LCHs, β-EP, and CRH exhibited the close relationship with $f_1$, and $f_2$, indicating that β-EP and CRH were the hubs of the master regulatory factors in the reproductive endocrine network and the regulator of hypothalamus itself. NOS the common molecule of $f_1$ and $f_3$, was the hub of the master regulatory factor in the reproductive endocrine network and the regulator of the hypothalamus acting on the pituitary-adrenal axis. CORT the common molecule of $f_3$ and $f_4$, was the hub of two regulators of the hypothalamus acting on the pituitary-adrenal axis.

## 4. Conclusion

Our factor analysis suggests that the master regulatory factor $f_1$ includes all four tested pituitary-ovary axis molecules and CORT in the target gland of the pituitary-adrenal axis following oral administration of KTHs, whose effect might then be transferred into the hypothalamus. $f_4$ regulator indicates the regulatory effects of the hypothalamus on the pituitary-adrenal axis following KTHs treatment. In conclusion, the effects of KTHs on the regulation of pituitary-adrenal axis-related molecules include direct interactions as well as indirect effects from the hypothalamus, similar to the overall mechanism of the DJD (Zhang et al, 2013). The hub of the master regulatory factors and other regulators, however, were quite different; The DJD targets IL-1 as the key biological molecule (Zhang et al, 2013), while KTHs target GnRH, consistent with a previous study of KTHs (Cai and Zhang, 2002) and a modern medical study of GnRH regulation by environmental factors (Ishwar Satoshi and Takashi, 2012). Interestingly, the master regulatory factor associated with LCHs treatment does not include effectors on the pituitary-adrenal axis, only $f_3$ and $f_4$ factors indicate the regulatory of the hypothalamus acting on the pituitary-adrenal axis for LCHs treatment. Thus, the indirect effects of LCHs on the pituitary-adrenal axis only account for the part of effects of DJD.

In conclusion, KTHs are fundamental components of the DJD formula, concordant with the idea that the DJD works by "tonifying the kidney" (Feng, 2012), while LCHs are shown to have more of a supporting role in POF treatment (Gao, 2010; Dong et al, 2010). Our study provides a more

comprehensive and thorough understanding of the biological mechanisms of DJD in clinical treatment and scientific studies.

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