Gene Cell Tissue. 2024 October; 11(4): e154371 https://doi.org/10.5812/gct-154371

Published Online: 2024 October 30 **Research Article**

# Evaluation of the Effects of Naringenin on the Hypothalamic mRNA Levels of *nesfatin*-1 and *CRH* in a Rat Model of PCOS

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**Received:** 7 September, 2024; **Revised:** 29 September, 2024; **Accepted:** 30 September, 2024

## Abstract

**Background:** Polycystic ovary syndrome (PCOS) is a common endocrine disorder and a leading cause of infertility in women. Studies suggest that naringenin may improve ovarian function; however, its molecular mechanism within the hypothalamus-pituitary-gonad (HPG) axis remains unclear.

**Objectives:** This study investigated the role of naringenin on the expression of *corticotropin-releasing hormone (CRH)* and *nesfatin-1* genes in the hypothalamus of PCOS rats.

**Methods:** Twenty rats, each weighing 180 - 200 g, were divided into four groups (n = 5). Polycystic ovary syndrome was induced by administering estradiol valerate (2 mg per rat). The control and PCOS groups received saline, while the other two PCOS groups received intraperitoneal injections of naringenin at doses of 20 and 50 mg/kg for 14 days. Hypothalamic samples were collected to measure gene expression via real-time PCR.

**Results:** The PCOS group showed a significant decrease in *CRH* and *nesfatin-1* gene expression compared to the control group ($P \leq 0.05$). Naringenin treatment significantly increased the expression of *CRH* and *nesfatin-1* genes in comparison to the PCOS group ($P \leq 0.05$).

**Conclusions:** Naringenin appears to have therapeutic potential in improving ovarian function in PCOS. Its effects are mediated through up-regulation of hypothalamic neuropeptides upstream of GnRH neurons.

*Keywords:* CRH, PCOS, Nesfatin-1, Naringenin

## 1. Background

Polycystic ovary syndrome (PCOS) is a common female disorder affecting women of reproductive age. PCOS is associated with irregular menstrual cycles, weight gain, hirsutism, and infertility. The exact cause of PCOS is not fully understood, but it is believed to involve a combination of genetic, hormonal, and lifestyle factors. Treatment for PCOS typically focuses on managing symptoms and addressing underlying hormonal imbalances (1). A key component of the hypothalamic-pituitary-gonad (HPG) axis, which governs reproductive function, is the control of gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. Disruption in this regulatory system can lead to hormonal imbalances and irregularities in the menstrual cycle, common features of PCOS (2). Neuropeptides may influence the development and progression of PCOS, and alterations in neuropeptide signaling within the hypothalamus may contribute to the dysregulation of GnRH secretion observed in women with PCOS (3).

Nesfatin-1 is a neuropeptide derived from the precursor protein nucleobindin-2 (NUCB2), consisting of 82 amino acids. It is distributed in the central nervous system, especially in the cerebral cortex, hypothalamus, pancreas, ovary, adipose tissue, and digestive system (4). Research has shown that nesfatin-1 can influence reproductive processes by acting on the HPG axis. Nesfatin-1 has been implicated in the modulation of GnRH/LH secretion, which plays a crucial role in the menstrual cycle and fertility. Evidence indicates that PCOS causes a reduction in the level of nesfatin-1 (5).

*Corticotropin-releasing hormone* (*CRH*) is a 41-amino acid peptide. The *CRH* neurons are located in the hypothalamus, particularly in the paraventricular

nucleus (PVN) (6). Research suggests that *CRH* may influence the pathophysiology of PCOS through its effects on the hypothalamic-pituitary-adrenal (HPA) axis, which controls the production and release of stress-related hormones such as cortisol. Dysregulation of *CRH* signaling may disrupt the balance of GnRH/LH, ovarian steroidogenesis, ovulation, and fertility in women with PCOS (7).

Naringenin is a flavonoid compound found in certain fruits, particularly citrus fruits such as grapefruits and oranges. This bioflavonoid exists in an inactive form (naringin) in plants, which is converted into its active form (naringenin) by bacteria belonging to the intestinal microbiome. Naringenin belongs to the class of phytochemicals and is structurally similar to 17β-estradiol, which has been associated with various health benefits (8). Its anti-inflammatory, insulin-sensitizing, anti-androgenic, and antioxidant properties make it a promising candidate for managing the symptoms and complications of PCOS (9).

## 2. Objectives

However, whether and how naringenin exerts its anti-PCOS effects is still unclear. Therefore, the aim of the present research was to investigate the role of naringenin in the regulation of hypothalamic *CRH* and *nesfatin-1* gene expression in a rat model of PCOS.

## 3. Methods

### 3.1. Animals

In the present study, adult female Wistar rats (180 - 200 g) were used. The rats were maintained in a laboratory under standard environmental conditions, with a temperature of 22 ± 2°C and a 12-hour dark/light cycle. They had free access to food and water.

### 3.2. Polycystic Ovary Syndrome Induction

A vaginal smear was performed to monitor the regular estrous cycle (proestrus, estrus, metestrus, and diestrus) for two weeks. To induce PCOS in the estrus phase, 2 mg of estradiol valerate dissolved in 0.2 mL of sesame oil was injected intramuscularly into each rat. Vaginal smears were then taken every 15 days. Finally, PCOS induction was confirmed 60 days after the estradiol valerate injection by examining vaginal cells under a light microscope. The presence of persistent cornified epithelial cells indicated the development of PCOS. Additionally, PCOS was confirmed by hematoxylin-eosin staining of ovarian tissue (Figure 1) (10).

### 3.3. Experimental Procedure

The rats were classified into four groups (n = 5). The injections were administered as follows: Group I served as the control and received 0.2 mL saline; Group II served as the PCOS group and received 0.2 mL saline; Groups III and IV received naringenin at doses of 20 and 50 mg/kg (IP), respectively, for 14 days.

### 3.4. Hypothalamic Sample Dissection

First, the animals were euthanized, and the skull was carefully opened to remove the brain. The hypothalamus region was identified according to the Watson-Paxinos atlas. The brain was positioned with the ventral surface facing up, and a 4 mm thick slice containing the hypothalamus was dissected (extending from the front near the optic chiasma, posteriorly to the mammillothalamic region, and laterally to the hypothalamic sulcus). The hypothalamic samples were then removed and immediately stored at -80°C.

### 3.5. Real-time Polymerase Chain Reaction

Total RNA was extracted from the tissue samples using TRIzol reagent. Reverse transcription from RNA to cDNA was then performed following the instructions provided with the cDNA synthesis kit (Biotech Rabbit, Germany). For RT-PCR, the SYBR Green master mix kit was used (Takara, Japan). The PCR protocol was set as follows: An initial cycle at 95°C for 900 seconds, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 10 seconds. The amplified products for *CRH*, *nesfatin-1*, and *GAPDH* were 103, 204, and 120 base pairs, respectively. Primer sequences are provided in Table 1. The $2^{-\Delta\Delta CT}$ formula was applied to calculate relative mRNA expression.

### 3.6. Statistical Analysis

SPSS software (version 16) was used to analyze the data. One-way ANOVA and Tukey's post-hoc tests were utilized to assess significant differences between groups. The findings are presented as mean ± SEM, with values considered statistically significant at $P \leq 0.05$.

## 4. Results

As shown in Figures 2 and 3, the induction of PCOS significantly reduced the mRNA levels of *nesfatin-1* and *CRH* compared to the control group ($P \leq 0.05$). Administration of 20 mg/kg naringenin did not result in a significant increase in the mRNA levels of *nesfatin-1 and*

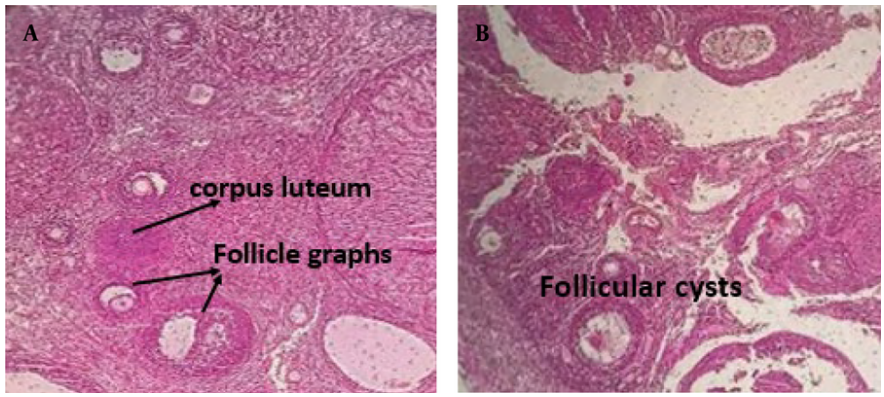


**Figure 1.** Ovarian histology. A, healthy ovaries; B, polycystic ovaries.

**Table 1.** Sequence of Sense and Antisense Primers

| Variables | Primers Sequences |
|---|---|
| ***nesfatin-1*** | |
| Sense | 5′- TGCAGAGAAGAACGCACCAG -3′ |
| Antisense | 5′- ACAGTACCGTGCTTGGATGG -3′ |
| ***CRH*** | |
| Sense | 5'- TGGATCTCACCTTCCACCTTCTG -3' |
| Antisense | 5'- CCGATAATCTCCATCAGTTTCCTG -3' |
| ***GAPDH*** | |
| Sense | 5′- AAGTTCAACGGCACAGTCAAG -3′ |
| Antisense | 5′- CATACTCAGCACCAGCATCAC -3′. |

*CRH* compared to the PCOS group. However, the injection of 50 mg/kg naringenin significantly increased the mRNA levels of *nesfatin-1* and *CRH* compared to the PCOS group ($P \leq 0.05$). Additionally, a significant difference was observed between the effects of 20 mg/kg and 50 mg/kg naringenin on the mRNA levels of *nesfatin-1* ($P \leq 0.05$).

## 5. Discussion

The consumption of herbal remedies has a positive association with the reduction of infertility problems. Previous studies have investigated the effect of naringenin on managing amenorrhea and abnormal ovulation. In the current study, *CRH* gene expression decreased in the PCOS group compared to the control. However, *CRH* mRNA levels returned to the baseline level of intact rats following naringenin injection for two weeks. Previous studies also indicate a decrease in *CRH* levels in PCOS patients compared to intact rats, supporting the present findings (11). *Corticotropin-releasing hormone* is synthesized in the hypothalamic paraventricular nucleus (PVN) and has various physiological functions, including the regulation of behavior, nutrition, reproduction, and GnRH neuron function (12). Evidence suggests that *CRH* is involved in steroid biosynthesis, inflammatory processes, ovulation, and luteolysis (13). In PCOS patients, the decrease in *CRH* gene expression may be due to increased androgen levels. The *CRH* neurons express androgen receptors (ARs), indicating that these neurons are targets for androgen action. The suppressive effects of androgens on *CRH* may occur directly by binding to ARs on *CRH* neurons in the PVN, or androgens may act on *CRH* neurons indirectly via the bed nucleus of the stria terminalis (BNST), the medial preoptic area (mPOA), the suprachiasmatic nucleus (SCN), and the arcuate nucleus (ARC). The PVN receives afferent signals from these

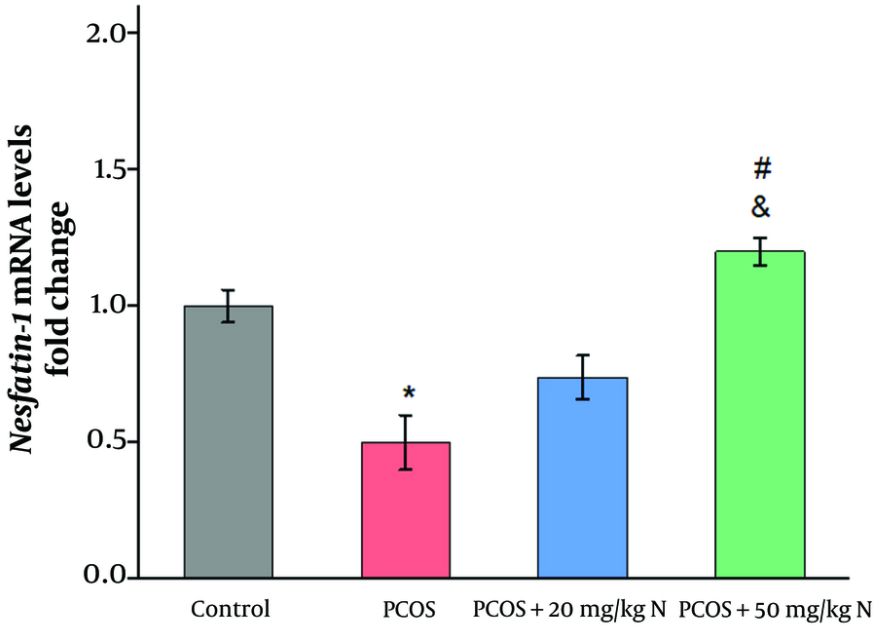


**Figure 2.** The effects of naringenin on the *nesfatin-1* gene expression in a rat model of PCOS. The results are expressed as mean ± SEM and significance was defined by $P \leq 0.05$. *: Compared with control; &: Compared to the PCOS group, #: Compared to the naringenin 20mg/kg group.

areas, which play a crucial role in regulating the HPA axis response (14).

In PCOS, the reduction of *CRH* may also be due to the effect of androgens on ghrelin. Androgen levels have an inverse relationship with ghrelin, as demonstrated by the increase in ghrelin levels following the injection of the anti-androgen flutamide (15). Ghrelin has a direct relationship with *CRH* neuron activity (16). This peptide is synthesized not only in gastric mucosal cells but also in specific populations of neurons within the central nervous system, including the hippocampus, hypothalamus, midbrain, and spinal cord (17). Research indicates that ghrelin's effect on GnRH secretion is mediated by the ghrelin receptor (GHS-R) located on GnRH neurons. When a ghrelin receptor antagonist is administered, it blocks the inhibitory effect of ghrelin on GnRH secretion (18). Additionally, women with PCOS have been reported to have lower ghrelin levels (19). Evidence suggests that ghrelin, by stimulating *CRH* neuron activity, promotes the release of *CRH*, adrenocorticotropic hormone (ACTH), and corticosteroids (16). Furthermore, previous findings indicate that naringenin enhances ghrelin receptor activity (20). Therefore, it is hypothesized that naringenin may contribute to the restoration of *CRH* levels to baseline in the PCOS model by stimulating the ghrelin receptor on *CRH* neurons.

Estrogen, a critical sex hormone in the reproductive axis, supports follicular growth and the secretion of gonadotropins from the pituitary gland. Estrogen acts through two receptors: the alpha receptor (ERα) and the beta receptor (ERβ). Studies show that estrogen levels decrease in PCOS due to the inhibition of aromatase activity, which is responsible for converting androgens to estrogen. This process is essential as estrogen plays a central role in ovarian function by regulating androgen production (21, 22). There is also an interaction between estrogen and *CRH* in reproductive regulation, as estrogen receptors are located on *CRH* neurons, and *CRH* is stimulated by both ERα and ERβ (23). Additionally, studies reveal that naringenin has structural similarities to beta-estradiol (24) and can bind to both alpha and beta receptors (25). It has also been reported that naringenin stimulates aromatase activity, potentially leading to an increase in estrogen levels (26). Thus, it is likely that naringenin increases *CRH* mRNA to baseline in the PCOS rat model by enhancing aromatase activity and estrogen receptor function.

In the present study, the effect of naringenin on hypothalamic gene expression of *nesfatin-1* was

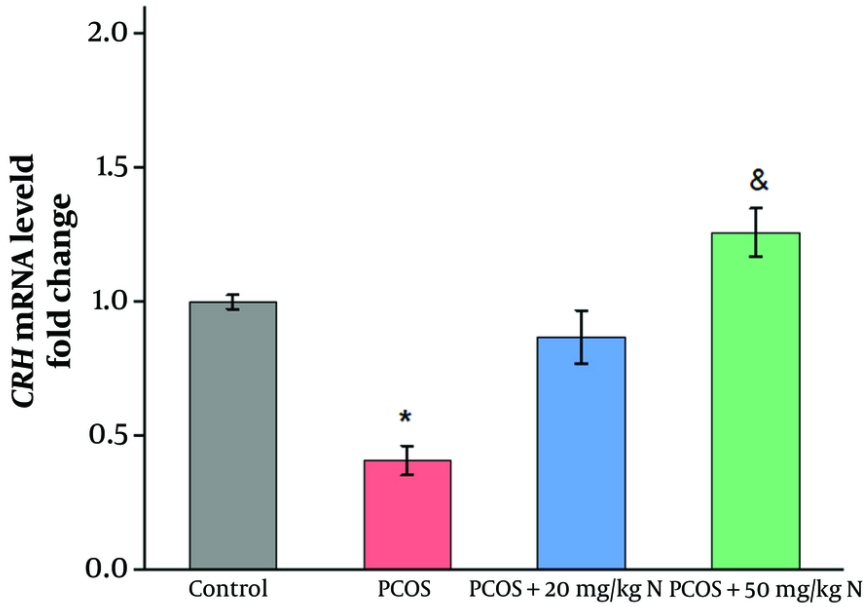


**Figure 3.** The effects of naringenin on the *CRH* gene expression in a rat model of PCOS. The results are expressed as mean ± SEM and significance was defined by $P \leq 0.05$. *: Compared with control; &: Compared to the PCOS group.

investigated in a rat model of estradiol valerate-induced PCOS. Results showed a significant decrease in *nesfatin-1* gene expression in the PCOS group compared to the control group, consistent with previous studies (27). Several studies in rats indicate the presence of *nesfatin-1* and its co-expression with other transmitters in the brain, particularly in hypothalamic nuclei such as the PVN and ARC (28). Evidence suggests that *nesfatin-1* can regulate reproductive function by acting directly on GnRH neurons (29). Research indicates a positive relationship between *nesfatin-1* and levels of follicle-stimulating hormone (FSH), estrogen, and progesterone. Low levels of *nesfatin-1* in PCOS impair follicular growth by inhibiting FSH (30).

One of the primary metabolic abnormalities associated with PCOS is insulin resistance (IR), which plays a significant role in the condition's pathophysiology (31). Many women with PCOS are overweight or obese, which exacerbates impaired insulin action (31). Elevated insulin levels can further reduce *nesfatin-1* levels in PCOS (32). As a natural compound, naringenin improves insulin sensitivity and glucose metabolism (33). In this study, naringenin administration increased hypothalamic gene expression of *nesfatin-1* in the PCOS model rats. The enhancing effect of naringenin on *nesfatin-1* may be mediated by its role in glucose homeostasis.

There is also a close relationship between the serotonergic system and *nesfatin-1*. Serotonin inputs in the hypothalamus interact with *nesfatin-1* neurons, with serotonin able to stimulate *nesfatin-1* production. For example, a study reported that administering a serotonin receptor agonist to mice increased *nesfatin-1* levels (34). Furthermore, previous research has shown that naringenin may influence serotonin system function, increasing serotonin levels in rats receiving naringenin (35). This suggests that naringenin may upregulate hypothalamic *nesfatin-1* expression by enhancing serotonergic system activity in the PCOS rat model.

### 5.1. Conclusions

In summary, naringenin increased the expression of *CRH* and *nesfatin-1* genes in the hypothalamus of PCOS rats. This study contributes to understanding the molecular mechanisms of naringenin in the reproductive system. Thus, naringenin, as a natural phytoestrogen, may help improve HPG axis function in PCOS.

## Acknowledgements

The authors appreciate the University of Mohaghegh Ardabili for supplying the required apparatus.

## Footnotes

**Authors' Contribution:** Literature search and data collection were performed by F. M. and Kh. H. The first draft of the manuscript was written by F. M., Kh. H., F. M. and Kh. H. supervised the work and Kh. H. conceptualized the study. All authors read and approved the final manuscript.

**Conflict of Interests Statement:** There is no conflict of interest in this article.

**Data Availability:** The dataset presented in the study is available on request from the corresponding author during submission or after publication.

**Ethical Approval:** The University of Mohaghegh Ardabili's Research Ethics Committee oversaw the study's execution (code: IR.UMA.REC.1403.001 ).

**Funding/Support:** University of Mohaghegh Ardabili supported this study.

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