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Study Association between Dopamine and *DRD2* Gene Expression of polycystic ovary Syndrome in Iraqi women

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Abstract:
Infertility stands as the most prevalent endocrine disorder among women of reproductive age. This study aimed to investigate the gene expression of D2 dopamine receptors and elucidate the altered levels of dopamine in infertile women compared to controls. Additionally, it sought to evaluate their involvement in infertility and their correlation with me (FSH), and prolactin using Enzyme-linked Immunosorbent Assay (ELISA) and Automated Immune Assay (AIA) kits. RNA extraction from whole fresh blood was followed by quantification and purity assessment using a nanodrop device. Subsequently, RNA was converted to cDNA for measuring DRD2 gene expression levels using Real-Time PCR.
Results: The study revealed a significant decrease in dopamine levels in infertile women compared to the control group ($P< 0.01$). Moreover, hormone level analysis indicated a substantial increase in prolactin (PRL) and luteinizing hormone (LH) levels among infertile females compared to controls ($P\leq0.01$), with no significant difference in follicle-stimulating hormone (FSH) levels. The fold expression of DRD2 was down-regulated in patients but up-regulated in the control group.
Conclusion**:** The findings suggest that gene expression of DRD2 and dopamine levels could serve as biomarkers for early detection of female infertility among Iraqi women, offering a potential laboratory diagnostic approach.
Keywords: Dopamine receptor, Infertile women, Prolactin, Luteinizing hormone, DRD*2* gene expression.

Introduction

Infertility is characterized by the inability to conceive following 12 months of regular unprotected sexual intercourse. Around 85% of couples experiencing infertility have an identifiable reason for their condition. The primary causes often include issues such as ovulatory dysfunction, male infertility, and tubal disease. The remaining 15% of cases are labeled as "unexplained infertility." Factors like lifestyle choices and environmental factors, such as smoking and obesity, can have adverse effects on fertility. Ovulatory disorders

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contribute to roughly 25% of infertility diagnoses, with 70% of women experiencing anovulation being diagnosed with polycystic ovary syndrome (PCOS), a complex condition affecting reproduction, metabolism, and psychological well-being throughout life. The underlying causes of PCOS are multifaceted and involve genetic and epigenetic susceptibilities, dysfunctions in the hypothalamus and ovaries, exposure to excessive androgens, insulin resistance, and mechanisms related to adiposity (1). Infertility can also be a marker of an underlying chronic disease associated with infertility, in addition to genetic causes and genetic developmental disorders, which play an important role in infertility in females (2,3). Dopamine plays a crucial role in various fundamental brain functions, including movement, behavior, cognition, motivation, and the secretion of hormones. Its effects are mediated through dopamine receptors, with dopamine D2 receptors particularly linked to reward mechanisms in the brain. When D2 dopamine receptors malfunction, it can result in abnormal seeking behaviors for substances. (4). Dopamine plays a role in regulating various physiological and behavioral functions, including reproduction, which in vertebrates is governed by the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus releases gonadotropin-releasing hormone (GnRH1), formerly known as luteinizing hormone-releasing hormone, stimulating the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. These gonadotropic hormones directly impact reproductive capacity by promoting the synthesis of gonadal steroid hormones such as testosterone, estrogen, and progestin.(5), Moreover, dopamine attaches to DRD2 receptors in the pituitary lactotrophs and reduces the amount of intracellular cyclic adenosine monophosphate, thereby suppressing the secretion of prolactin.(6)

Materials and methods:

Sample Collection: This observational study involved 50 infertile females as patients and 50 controls. Blood samples (5ml) were obtained using EDTA tubes. From each EDTA tube, 250µl of blood was combined with 750µl of Triazol in Eppendorf tubes, which were then stored in a deep freezer (-20 ºC) for molecular analysis. The remaining samples were centrifuged, and the serum was collected and stored in a refrigerator (4 ºC) for ELISA and AIA biochemical tests. Hormonal examinations were conducted to measure serum concentrations of dopamine, prolactin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH).

RNA extraction was performed using the TransZol Up Plus RNA Kit Reagent as per the manufacturer's instructions. The purity and concentration of RNA were assessed using a spectrophotometer (Nanodrop). Subsequently, cDNA synthesis from mRNA was carried out. The expression levels of the DRD2 gene were evaluated using the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, known for its sensitivity in

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quantifying steady-state mRNA levels. A quantitative real-time qRT-PCR SYBR Green assay was employed to confirm the expression of the target gene. The mRNA levels of the endogenous control gene GAPDH were amplified and used to normalize the mRNA levels of the DRD2 gene.

RNA concentration and purity assessment

The concentration and purity of the extracted RNA were assessed using the NanoDrop One C (Thermo Fisher Scientific, USA). This instrument measured RNA concentrations ranging from 73 to 147 ng/µl. The purity of the RNA samples was determined by measuring absorbance at two wavelengths (260 and 280nm). An A260/A280 ratio close to 2.0 indicated the purity of the RNA sample.

Synthesis the cDNA form mRNA

For the first strand cDNA synthesis, the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit were utilized to reverse transcribe total RNA into complementary DNA (cDNA). Following the manufacturer's protocol, the reaction was carried out in a volume of 20 µl. Specifically, 4µl of total RNA was subjected to reverse transcription. The primers designed for the study are detailed in (Table 1).

Quantitative Real Time PCR (qRT–PCR)

The expression levels of the *DRD2* gene were estimated by the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, a sensitive technique for quantifying of steady-state mRNA levels. To confirm the expression of the target gene, a quantitative real-time qRT-PCR SYBR Green assay was used. Alpha DNA Ltd. (Canada) designed and synthesized primer sequences for the *DRD2* gene, then lyophilized and stored at -20°C (Table1)

(Table 1): The components of quantitative real-time PCR were employed in the ***GAPDH and*** *DRD2*gene expression experiments.

Components	20 µl rxn
2xTransStart® Top Green qPCR Super Mix	10
Nuclease free water	4

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Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
cDNA	4

The endogenous control gene *GAPDH*'s mRNA levels were amplified and utilized to normalize the *DRD2* gene's mRNA levels. (Table2) shows the primer sequences for *GAPDH* and *DRD2* genes. The cycling protocol was programmed for the following optimized cycles and according to the thermal profile shown in (Table 4).

Table (3): The study's designed primers.

Primer	**Sequence (5′→3′ direction)**	**primer size bp**	**Product size bp**	**Ta °C**
	DRD2 (Gene Expression)			
Forward	CTGCAGACCACCACCAACTA	20	154	58
Reverse	TGACGTCCAGAGTGACGAAG	20		
***GAPDH*- Glyceraldehyde 3-phosphate dehydrogenase**				
Forward	GAAATCCCATCACCATCTTCCAGG	24	160	58
Reverse	GAGCCCCAGCCTTCTCCATG	20		

Table (4): The thermal profile of ***GAPDH and DRD2*** gene expression**.**

Step	**Temperature (°C)**	**Time (sec.)**	**Cycles**
Enzyme activation	94	10	1
Denaturation	94	5	40
Annealing	58	15	
Extension	72	20	
Dissociation	55 °C-95 °C		1

Statistical analysis was carried out using SPSS version 23. Categorical

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variables were presented as frequencies and percentages. The chi-square test and Fisher exact test were used to compare percentages (frequencies) in this study. The odds ratios (ORs) and 95% confidence intervals (95%CIs) were used to evaluate the potential associations between gene expression of dopaminergic genes and the risk of infertility *P* value for all tests was considered significant if ≤ 0.05

Results and Discussion

Serum Dopamine: infertile females compared with healthy women showed significant decreasing in Dopamine level (13.4966±2.53960 vs 22.3652±15.62011.; P≤0.01) (table 5) Generally, the results of the present study agree with Wasilewski et al (7) who found alters dopamine metabolism may cause menstrual disturbances as well as heighten the risk of miscarriage by decreased dopamine levels, which may lead to increased prolactin concentration followed by hyperprolactinemia, which causes ovulation disturbances, changes in the luteal phase as well as amenorrhea or oligomenorrhea .also, dopamine may slow down the pulsation of gonadotropin-releasing hormone, causing an increase in luteinizing hormone levels. Furthermore, decreased DA concentrations along with reduced dopamine 2 receptors. They attributed low DA concentrations to the increase in LH concentrations in infertile females. inhibitory neurotransmitters in hypothalamic-pituitary center. This hypothesis, is responsible for the depression and anxiety-like mood disorders commonly seen in PCOS women.(8)

Table(5): The Dopamine (pg/ml) in patients and control groups.

Groups	Mean(pg/ml)	Std. Deviation	Std. Error of Mean	p-value
Patients	13.4966	2.53960	.35561	0.001**
Control	22.3652	15.62011	3.59669	
Total	16.3345	10.87201	1.25539	

Serum luteinizing hormone (LH): There were significantly elevated increase in serum prolactin concentrations and LH in infertile females compared to healthy women showed significant increase in PRL (22.226 ±8.7353 vs. 12.446 ±2.26290; P≤0.01), and significant increase in LH (6.2466±3.819400 vs. 3.9680 ±.92327; P≤0.01) (table 6).

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Table (6): The Serum luteinizing hormone (mlU/ml) in patients and control group.

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Patients	6.2466	3.81940	.54015	0.0001**
Control	3.9680	.92327	.13057	

These results were agree with Al-Juaifari and Al-Jumaili (9,10) who was found that LH level increase in infertile females compared to healthy control, especially in PCOS females .as result of the heterogeneity of infertility, there are most likely multiple underlying pathophysiologic mechanisms, which causes alteration in gonadotropin-releasing hormone secretion results in increased luteinizing hormone (LH) secretion. Also, high levels of LH not only has an effect on oocyte maturity and human reproduction but also on lower fertility and higher miscarriage prevalence(11) .

Serum Prolactin The serum prolactin concentrations in infertile females were (22.226 ±8.7353) compared to healthy women 12.446 ±2.26290; P≤0.01), (table 7)

Table (7): The serum prolactin value (ng/ml) in patients and control groups.

Groups	Mean(ng/ml)	Std. Deviation	Std. Error of Mean	p-value
Patients	22.226	8.7353	1.2354	0.0001**
Control	12.446	2.2629	0.3200	

Hyperprolactinemia in infertility women might contribute to obesity, hyper insulinemia, and gonadal dysfunctional in infertile females furthermore, hyperprolactinemia might be linked with increased risk of metabolic syndrome and probably become a metabolic risk. In women,

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it frequently leads to gonadal dysfunction including ovulatory disorder, menstrual galactorrhea and infertility (12,13).

Serum follicle stimulating hormone (FSH): The serum follicle stimulating hormone (FSH) was (5.1442 ±3.28634) compared to healthy control females (5.3180±.99297) ($p>0.05$) table (8).

Table(8) The Serum follicle stimulating hormone (FSH) (mlU/ml) in patients and control groups.

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Patients	5.1442	3.28634	.4647600	0.7
Control	5.3180	0.99297	0.14043	
Total	5.2311	2.41684	0.24168	

Yarmolinskaya *et al*., (14) found that the low level of FSH and higher LH hormones in infertile females led to increased LH/FSH ratio associated with less response for progesterone and defect in the FSH, and that was associated with poor ovulatory response (9).

Furthermore, Al Faisal and Al-Deresawi (15) were reported that results obtained from the hormonal analysis showed significantly lower levels FSH in infertile women. High levels of luteinizing hormone (LH) and low levels of the follicular-stimulating hormone (FSH), so follicles in these individuals are prevented from producing a mature egg which was considered a main causes of infertility.

Fold expression of *DRD2* gene

In the present study, quantitative RT-PCR assay analyzed the mRNA expression of *DRD2* and compared its expression between infertile women *versus* apparently healthy control groups. The calculation of gene expression fold change was made using relative quantification (16).

The gene expression Fold of infertile females was (0.6791) and for apparently healthy control was (1.00) (table 9). When calculating the gene expression was lower in women with infertility than apparently healthy control (Figures 1,2) show the amplification plots and dissociation curves for *DRD2* and *GAPDH* Genes

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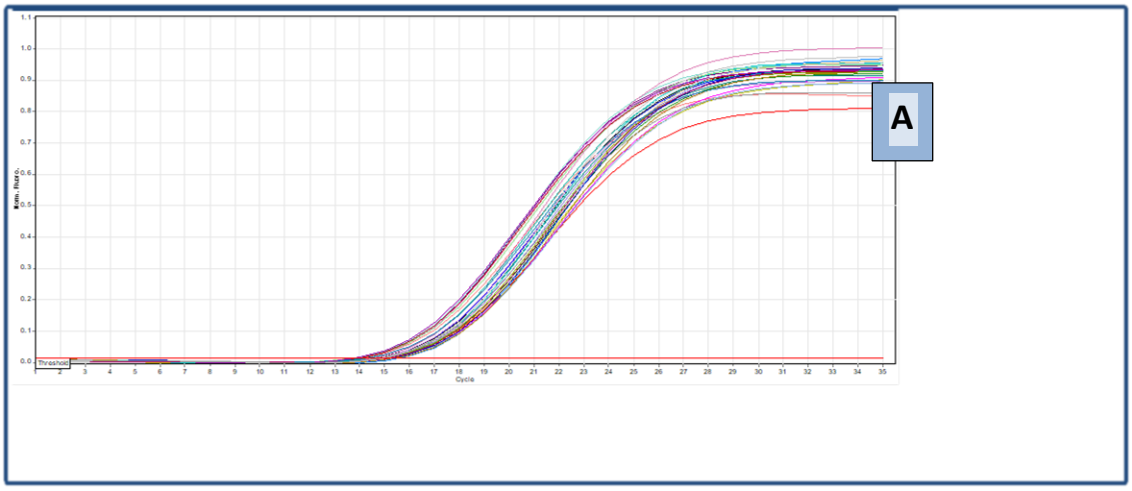
Table (9): The Gene expression comparison between patients and control groups.

groups	*Means Ct of DRD2*	*Means Ct of GAPDH*	*ΔCt (Means Ct of DRD2)*	*$2^{-\Delta Ct}$*	*experimental group/ Control group*	*Fold of gene expression*
Patients	27.8373	14.7944	13.0433	0.000118	0.000118/0.000191	0.6791
Control	27.1066	14.7593	12.3473	0.000191	0.000191/0.000191	1.00



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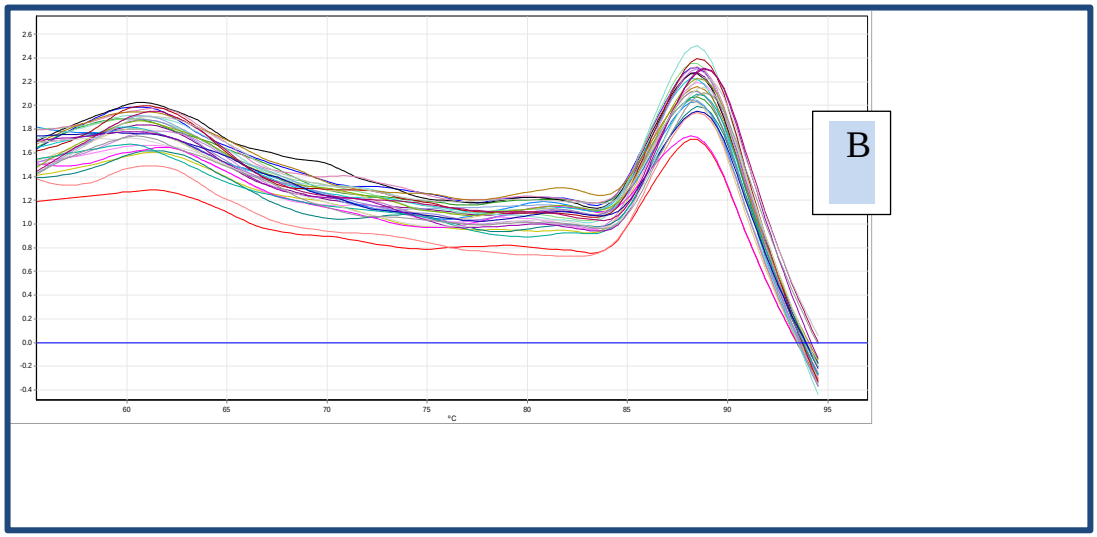
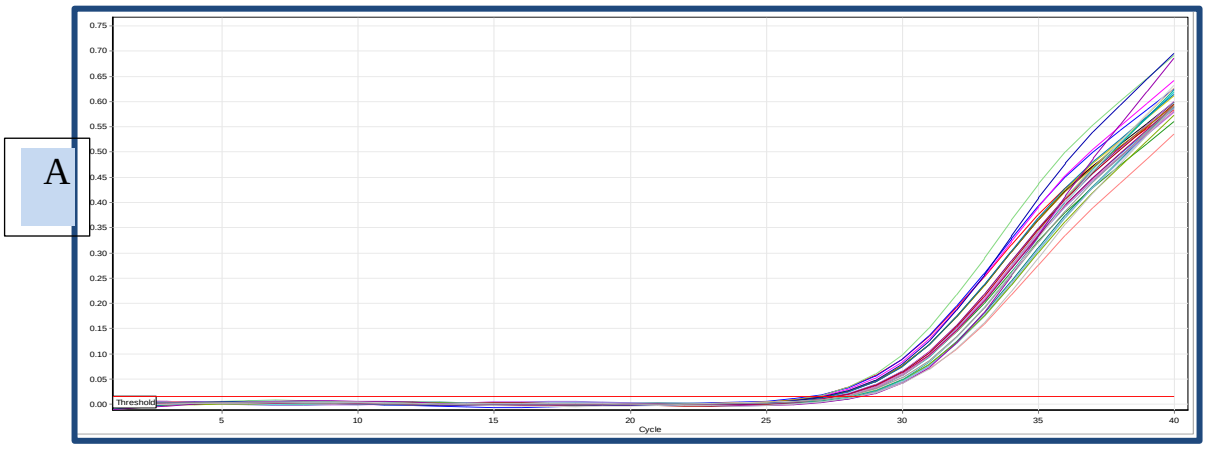


Figure (1): A-*GAPDH* gene amplification was plotted using qPCR samples that covered all research groups.. **B- *GAPDH*** gene dissociation curves using qPCR samples that covered all research groups. Melting temperatures varied from 81°C to 83°C. The images were captured using the Qiagen Rotor Gene Q qPCR apparatus.



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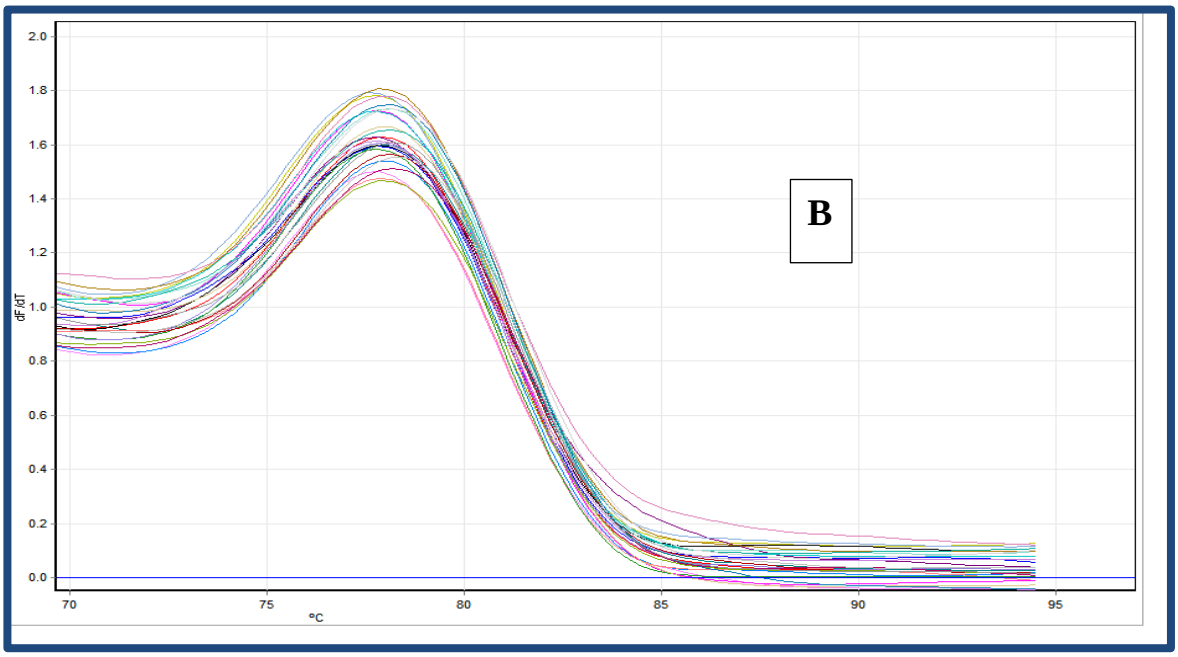

. **Figure (2): A-** ***DRD2*** gene amplification was plotted using qPCR samples that covered all research groups.**B-** ***DRD2*** gene dissociation curves using qPCR samples that covered all research groups. Melting temperatures varied from 78°C to 81°C. The images were captured using the Qiagen Rotor-Gene Q qPCR apparatus.

Markedly reduced D2R expression, which could lead to hyper prolactin production in female infertiles, particularly those with PCOS. which Numerous studies that corroborate our findings point to the function of elevated LH release and decreased dopaminergic tone in female infertility. Furthermore, PCOS patients who get treatment with the D2 receptor agonist bromocriptine can resume a regular menstrual cycle and ovulation (7,17). Additionally, it was noted that infertile females had decreased Drd2 expression and increased vascularization in the theca layer of antral and luteinized follicles. Decreased dopamine synthesis and diminished cabergoline's ability to block VEGF secretion Higher VEGF and vascularization, which increase the risk of ovarian hyperstimulation syndrome, might be explained by decreased dopaminergic tone and dysregulated Drd2 signaling (18, 19). Yarmolinskaya et al. (19) also reported DRD2 expression.

Significantly decreased in the expression of *D2R*, which may result into hypersecretion of prolactin in infertile females especially in PCOS condition. which, Supporting our data, many studies suggest the role of reduced dopaminergic tone increased LH releasein in infertile females Additionally, treatment with bromocriptine, a D2 receptor agonist, can restore normal

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menstrual cycle and ovulation in PCOS women (7,17). Furthermore, the Decreased of *Drd2* expression and increased vascularization in the theca layer of antral and luteinized follicles in infertile females were observed. Lower dopamine production and reduced efficacy of cabergoline in inhibiting VEGF secretion Decreased dopaminergic tone as well as deregulated *Drd2* signaling explain higher VEGF and vascularization leading to increased ovarian hyperstimulation syndrome risk (18),as well as Yarmolinskaya *et al* (19) reported *DRD2* expression was significantly downregulated in endometriosis areas, and there was a negative association between *DRD2* and PRL levels as well as a decrease in dopaminergic modulation, which led to an elevation of local PRL levels and VEGF in female infertile. Conversely, higher DA concentrations led to a rise in free radical generation, which decreased cell viability and adenosine triphosphate levels. These effects were linked to poor embryo quality, decreased oocyte maturation and fertilization, and a drop in pregnancy rates (12).

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