



Original Research

The Influence of Vitamin D3 Supplementation on LH and FSH Levels in Female Rats (*Rattus norvegicus*) with Polycystic Ovary Syndrome (PCOS) Model

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KEYWORD

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ABSTRACT

Introduction: Polycystic Ovary Syndrome (PCOS) is a common hormonal disorder in reproductive-aged women, characterized by hormonal imbalances such as Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). This study investigates the impact of Vitamin D3 supplementation on LH and FSH levels in female rats with a PCOS model.

Material and Methods: In this laboratory experiment, female rats were induced with testosterone propionate for 28 days. They were divided into three groups: negative control, positive control (testosterone only), and treatment (testosterone + Vitamin D3 supplementation). LH and FSH levels were measured using the enzyme-linked immunosorbent assay method.

Results: The results revealed that vitamin D supplementation significantly reduced FSH levels in the treatment group compared to the positive control while it increasing FSH levels. Regression analysis demonstrated a negative correlation between Vitamin D supplementation and LH levels, as well as a positive correlation with FSH levels.

Conclusion: Vitamin D3 supplementation appears to have a regulatory effect on LH and FSH levels in female rats with PCOS. Future research could explore the impact of higher Vitamin D3 doses and conduct further investigations at advanced stages of PCOS. These findings contribute to our understanding of PCOS and potential interventions involving Vitamin D3.

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INTRODUCTION

World Health Organization (WHO) estimated that around 116 million women (3.4%) in 2012 experienced PCOS worldwide. Incidence figures based on the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine show that the prevalence of PCOS in the world population is 15-20%. Meanwhile, according to Deswal R et al., in 2020, PCOS affected around 4-20% of women of childbearing age worldwide [1]. In Indonesia, based on research conducted by Goh et al.

(2022), it was found that the highest frequency of PCOS incidence was in the 26-30 year age range, namely 45.7% [2].

PCOS occurs due to complex interactions between genetic and environmental factors. Insulin resistance and LH hypersecretion are the main factors proposed in the pathogenesis of ovulation disorders and hyperandrogenism in PCOS. Hyperinsulinemia that occurs due to insulin resistance will then cause a state of hyperandrogenism through an increase in the activity of the pituitary-pituitary-adrenal axis, thereby increasing the activity of the 17 α -hydroxylase enzyme in the theca cells and a decrease in the production of sex

hormone binding globulin (SHBG) in the liver so that levels of free testosterone will increase. LH hypersecretion occurs due to negative feedback from the ovaries, especially the theca cells, to the anterior pituitary, which then causes hypothalamic "perception" errors that respond to increased GnRH pulsatility. This causes the cycle LH to be more dominant and FSH to be suppressed. This will cause the follicle to be unable to become a dominant follicle that ovulates. The absence of ovulation causes a condition of "unopposed estrogen" due to the lack of formation of the corpus luteum, which produces progesterone [3, 4].

Giving adjuvant therapy with vitamin D supplementation has been widely studied to improve the phenotype of patients with PCOS. Vitamin D deficiency is common in 70-85% of women with PCOS with serum 25-hydroxy vitamin D (25OHD) levels <20 ng/ml. Vitamin D is a fat-soluble vitamin that contains a steroid molecular structure. Vitamin D has been studied to have effects on controlling infections, hormonal regulation, and stimulating the differentiation of various cell types. Because of its various advantageous effects, vitamin D is considered to have a place in the management of PCOS [5].

Other recent research states that low levels of 25(OH)D can cause insulin resistance and hyperinsulinemia, contributing to ovulatory dysfunction in PCOS. Research on female Wistar rats shows that vitamin D supplementation improves follicular normality, increases concentrations of the hormones FSH and estradiol, and reduces concentrations of testosterone, luteinizing hormone, glucose, insulin, and insulin resistance in rats with PCOS [6].

Based on the background above, this experimental study will look for the role of vitamin D supplementation in managing patients with PCOS, significantly increasing LH levels and decreasing FSH in animal models of PCOS. Although larger-scale randomized controlled trials are needed, this study aims to be valuable as additional literature in understanding the effects of vitamin D supplementation in PCOS patients.

MATERIAL AND METHODS

Study Design and Population

This research employed a pure experimental design in a laboratory setting in vivo, using a Post Test Only Controlled Group Design to investigate the differences in Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) levels in female Wistar albino rats (*Rattus norvegicus*). The study was conducted at the Laboratory of Experimental Animals (LPHC) and the Parasitology Laboratory, Faculty of Medicine, University of Brawijaya, Malang, from August 2022 to October 2022.

The target population for this research consisted of female Wistar albino rats (*Rattus norvegicus*). The research sample comprised 24 female Wistar albino rats, aged 2 months, with a body weight ranging from 200 to 250 grams. These rats were obtained from the Parasitology Laboratory, Faculty of Medicine, University of Brawijaya, and underwent a one-week acclimatization period. To determine the sample size, the Frederer formula was used. The result showed that a minimum of 8 rats were used for each group, resulting in a total of 24 subjects.

The inclusion criteria for this study encompassed female Wistar albino rats (*Rattus norvegicus*) of approximately 2 months old, with a body weight ranging between 200 and 250 grams, and in overall healthy condition, displaying active behavior without any anatomical abnormalities. Additionally, the rats selected for the study had not been previously utilized in any other research investigations. Conversely, the exclusion criteria comprised pregnant rats, those with significant and sudden weight loss, rats exhibiting signs of illness characterized by inactivity, and any rats that had unfortunately passed away prior to or during the study.

The rats were acclimatized for a period of 7 days in the Laboratory of Experimental Animals (LPHC) at the Faculty of Medicine, University of Brawijaya (FKUB). The purpose of this acclimatization period was to facilitate their adaptation to the laboratory environment, including both the cages and their diet. Throughout the research, the rats were housed in plastic cages measuring 50x30 cm, with a bedding of wood shavings that was replaced every 4 days. They were provided with a standard diet and had access to water ad libitum. These cages were placed in a naturally ventilated room with exposure to natural light conditions.

Identification of the Estrus Cycle

Vaginal smears were obtained using cotton buds, cover glass, Giemsa stain, methanol, physiological saline (NaCl), and a microscope. Cotton buds dipped in physiological saline were gently inserted into the vaginal opening and rotated to collect mucus. The collected mucus was then spread onto a glass slide, fixed with methanol, and stained with Giemsa for 15 minutes. After Giemsa staining, a cover glass was placed, and the vaginal smear was observed under a microscope to determine the estrus phase.

Dosage and Administration of Testosterone Propionate for the PCOS Rat Model

The PCOS rat model was created by intramuscularly inducing testosterone propionate at a dosage of 1 mg/100 g body weight once daily for 28 days, following the method outlined by Wulandari in 2017 [7].

Table 1. Comparison of LH Levels

Group	Luteinizing Hormone (LH) level (Mean \pm SD)	p-value
Negative Control	2.8288 \pm 1.1099 ^a	0.000
Positive Control	6.315 \pm 1.5198 ^b	
Intervention Group	3.0275 \pm 0.8846 ^a	

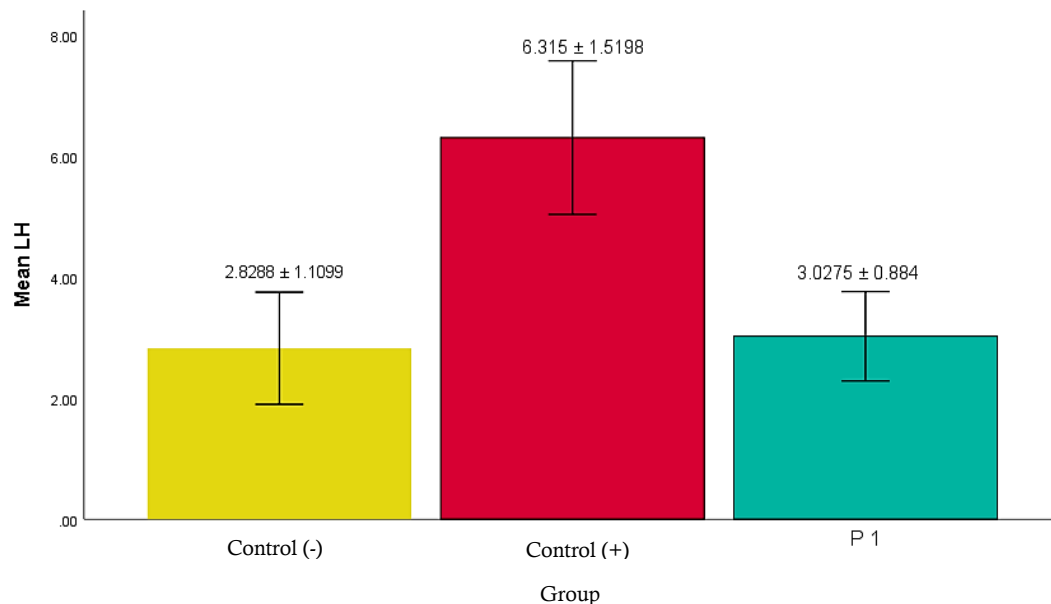


Fig. 1. Histogram of Mean LH Levels. Illustrates the histogram depicting the mean LH levels across all control and treatment groups. It can be observed that the positive control group has the highest average LH levels. Meanwhile, the lowest LH levels are exhibited by the negative control group.

Preparation and Administration of Vitamin D

Cholecalciferol was weighed, approximately 1 mg, and then dissolved in 100 mL of propylene glycol. This solution served as the primary stock solution, which was subsequently diluted to the desired dosage for administration to the rats. Cholecalciferol was administered orally once a day for 14 days. The chosen dosage was 25 mcg/kg body weight. The rats' conditions were monitored daily, and their body weights were recorded weekly. This dosing regimen was determined based on the vitamin D therapeutic index for experimental rats.

Termination of Experimental Animals

Anesthesia was administered to both the control and treatment groups 48 hours after the last treatment. Prior to anesthesia, the rats underwent a 10-hour fasting period. Anesthesia was induced using approximately 5 mL of chloroform per rat, which was poured onto cotton and placed inside an anesthesia chamber. Within approximately 1 minute, the rats ceased breathing, as indicated by dilated pupils and immobility of their limbs.

Blood Sample Collection from Experimental Animals

On the final day of the experiment, the rats were fasted for 10 hours before blood samples for LH and FSH measurement were collected. Approximately 3 mL of blood was obtained via intracardiac puncture through the heart's apex.

Measurement of LH and FSH Levels in Experimental Animal Blood

Blood samples collected from the rat's heart were transferred to reaction tubes and allowed to stand for 30 minutes to 3 hours until serum separation occurred. Subsequently, the serum was centrifuged at 3000 rpm at room temperature for thirty minutes using a Hettich Zentrifugen centrifuge. The centrifugation process was carried out to obtain pure serum. The obtained serum was then transferred to Eppendorf tubes, and LH and FSH levels were promptly examined using the ELISA method with ELISA kits from BT-LAB.

Table 2. Comparison of FSH Levels

Group	Luteinizing Hormone (LH) level (Mean ± SD)	p-value
Negative Control	10.8325 ± 2.0203 ^a	0.000
Positive Control	10.2675 ± 1.3927 ^a	
Intervention Group	13.2075 ± 1.0241 ^b	

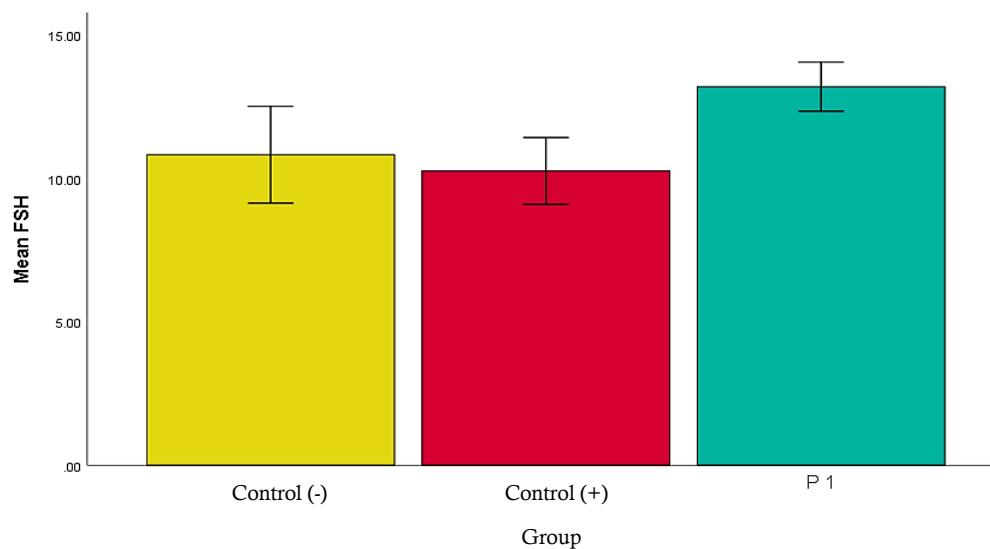


Fig. 2. Histogram of Mean FSH Levels. Illustrates the histogram displaying the mean FSH levels across all control and treatment groups. It can be observed that the intervention group has the highest average FSH levels. Meanwhile, the lowest FSH levels are exhibited by the positive control group.

RESULTS

Statistical Analysis

Comparison of LH Levels

The results of the investigation regarding the influence of vitamin D3 supplementation on Luteinizing hormone (LH) levels are presented in Table 1. On the mean ± standard deviation, if different letters are present, it indicates a significant difference ($p < 0.05$), whereas if the same letters are present, it shows no significant difference ($p > 0.05$).

Based on Table 1, the analysis results, it can be concluded that there is a significant influence of vitamin D3 supplementation on LH levels. The lowest average LH level was observed in the negative control group, at 2.8288 ± 1.1099 , and there was no statistically significant difference with the intervention group. This demonstrates that vitamin D3 supplementation is capable of reducing LH levels in the intervention group (Fig. 1).

Comparison of FSH Levels

The results of the investigation on the effect of vitamin D3 supplementation on FSH levels using ANOVA. Based on Table 2, the analysis results, it can

be concluded that there is a significant influence of vitamin D3 supplementation on FSH levels. The administration of vitamin D3 in the intervention group resulted in an FSH level of 13.2075 ± 1.0241 , and there is a statistically significant difference when compared to the negative control and positive control groups. This demonstrates that vitamin D3 supplementation in the intervention group is capable of increasing FSH levels (Fig. 2).

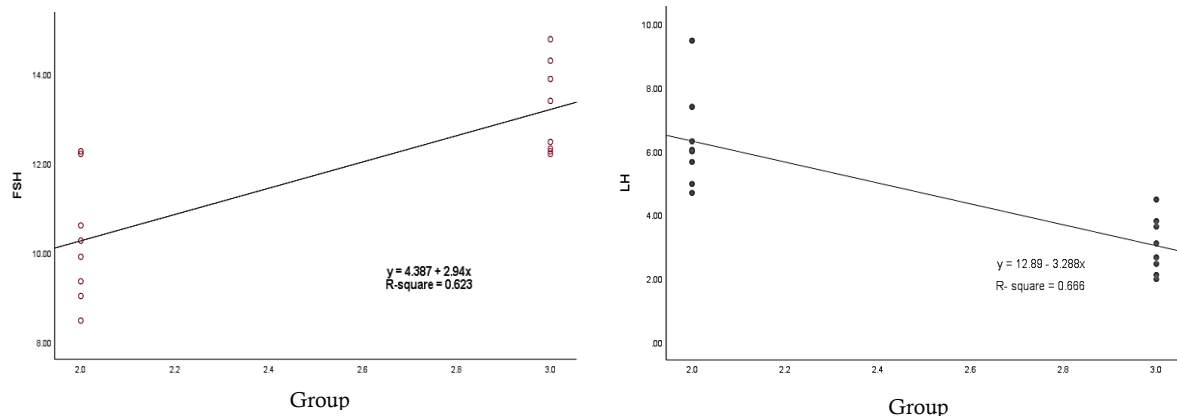
Regression Analysis of the Effect of Vitamin D3 on LH and FSH Levels

Based on Table 3, to determine the impact of vitamin D3 supplementation on LH and FSH levels, regression analysis can be conducted. Based on the regression analysis results, it is evident that the administration of vitamin D3 can reduce LH levels by 66.6%. The remaining 23.4% is attributed to other factors not involved in the study. The regression coefficient of -3.288 signifies that an increase of 1 dose of vitamin D is capable of reducing LH levels by 3.288. Additionally, the administration of vitamin D3 can influence an increase in FSH levels by 62.3%, with the remaining 37.7% attributed to other unexamined factors (Fig. 3).

Table 3. Regression Analysis of the Effect of Vitamin D3 on LH and FSH Levels

Variabel	Regresion Frormula	p-value	Coefficient Correlation and p-value	R-Square
FSH	$Y1 = 4.387 + 2.94X$	0.000	$r = 0.789$ p = 0.000	62.3%
LH	$Y2 = 12.89 - 3.288X$	0.000	$r = -0.816$ p = 0.000	66.6%

Y1; FSH expression, LH expression, X, vitamin D supplementation

**Fig. 3.** Scatter Plot of the Relationship between Vitamin D3 Supplementation and LH and FSH Levels

DISCUSSION

The Relationship Between Vitamin D3 and LH Levels in the PCOS Rat Model

It has been explained that PCOS is a complex systemic metabolic disorder involving insulin resistance, hyperandrogenism, and hypersecretion of LH. Vitamin D deficiency has long been associated with metabolic disturbances in the body, particularly related to glucose and insulin metabolism, as well as disruptions in steroidogenesis. Vitamin D, as an endocrine system regulator, possesses the ability to control infections, autoimmune diseases, and organ transplant tolerance.

This is based on the capabilities of 1,25(OH)₂D₃, which has pro-differentiation effects on monocyte macrophages, antigen-presenting cells (APCs), dendritic cells (DCs), and lymphocytes. 1,25(OH)₂D₃ also acts as an adjuvant for vaccines, with its mechanism involving the induction of p21 and C/EBP β , which mediate the enhancement of macrophage-monocyte immune function. Furthermore, 1,25(OH)₂D₃ induces p21, which plays a direct role in the differentiation of monocytes into mature macrophages. C/EBP β is a crucial transcription factor for macrophages, serving as an antibacterial, antiviral, and antitumor agent, and is also vital for the synthesis of IL-12, a cytokine that mediates Th1 immune function [8].

The hypersecretion of LH in PCOS can be explained through two mechanisms. The first theory explains that disruptions in the secretion of GnRH occur due to

disturbances in gonadotropin-inhibitory hormones (GnIH), primarily RFamide-related peptide-3 (RFRP-3), which is a neuropeptide found in mammalian animals and located in the dorsomedial hypothalamus. Disturbances in these inhibitory hormones result in disruptions in LH pulsatility, causing LH to be continuously secreted throughout the cycle. The subsequent theory attributes the hyperandrogenic conditions to feedback disruptions. Hyperinsulinemia influences cytochrome P450 in the ovaries, which plays a role in aromatization processes, leading to the accumulation of testosterone and DHEAS.

The continuous increase in androgen levels provides positive feedback on LH levels, causing LH secretion to remain elevated while FSH levels are suppressed. Follicular growth continues, resulting in the accumulation of antral follicles, but they never reach the dominant follicle stage capable of ovulation. Hyperandrogenism leads to follicular atresia, followed by antral follicle arrest, ultimately resulting in infertility in PCOS. Approximately 75% of PCOS patients exhibit hyperandrogenism, with over 80% of them having elevated free testosterone levels above normal [9].

Vitamin D plays a crucial role in women's reproductive function, particularly in its involvement in the steroidogenesis process. Vitamin D acts as a co-factor in the conversion of cholesterol into a significant amount of androgens. Therefore, the effects of vitamin D deficiency on ovulatory function in women with PCOS are related to the hypersecretion of LH due to negative

feedback from the ovaries, specifically the theca cells to the anterior pituitary, subsequently causing a "perception" error in the hypothalamus, leading to an increased pulsatility of GnRH. This results in LH dominance throughout the cycle, with FSH being suppressed.

Follicles under the influence of dominant LH will experience arrested development at the antral follicle stage with thicker theca cells, rendering them unable to become dominant follicles capable of ovulation. Additionally, vitamin D is known to have immunomodulatory effects, protecting oocytes from oxidative damage. Furthermore, vitamin D3 is a co-factor that regulates calcium flow through the membranes of beta cells in the pancreas and insulin targets in peripheral tissues. It is also known that vitamin D stimulates insulin receptors to enhance insulin targeting for glucose transport and has a direct effect on cytokines to reduce systemic inflammation, which in turn can reduce ovarian androgen production and increase SHBG [10].

In our research, we found that the administration of vitamin D3 at a dose of 25 µg/day significantly reduced the levels of LH in rats induced with 1 mg/100 g BW of Testosterone Propionate and subsequently given vitamin D3 compared to the group of rats induced with only 1 mg/100 g BW of Testosterone Propionate. This is in line with a previous study conducted by Besmanesh et al. in 2019, where the administration of vitamin D3 to Wistar rats with a PCOS model significantly reduced LH levels compared to the control group. Therefore, treatment with vitamin D in rats with PCOS significantly reduces LH concentration [6].

Consistent data from other studies also indicate that vitamin D deficiency correlates with an increase in gonadotropin levels, especially LH. Hence, in the future, controlled randomized trials to determine the effective therapeutic dose and toxic levels are essential. This will help determine whether correcting vitamin D deficiency, especially in PCOS patients, can improve reproductive function in PCOS patients [11].

The Relationship Between Vitamin D3 and FSH Levels in PCOS

Due to the dominance of LH throughout the cycle, follicles become insensitive to FSH. Suppression of FSH also occurs due to disruptions in VDRE (Vitamin D Response Element) in granulosa cells, where under normal conditions, vitamin D binding activates VDRE genes, allowing FSH to bind to its receptors and initiate the process of aromatizing androgens into estradiol. Vitamin D deficiency prevents the conversion of active androgens into estradiol. Additionally, in cases of hyperinsulinemia, insulin indirectly enhances the inhibin mechanism, suppressing FSH production, while LH production remains high throughout the cycle [12].

Numerous studies have shown that vitamin D3 supplementation can mitigate the adverse effects of PCOS. Observations on the ovaries of PCOS model rats by Besmanesh et al. in 2019 revealed a significant number of cysts and damaged follicles at various developmental stages, with atrophic granulosa cells and hypertrophic theca layers. In this study, the percentage of atretic follicles at various developmental stages decreased in rats with PCOS, disrupting follicle growth and folliculogenesis, and ultimately leading to a lack of ovulation during development [13].

In PCOS patients, there is an increased frequency and amplitude of GnRH pulsation, which is higher in women with PCOS compared to normal women. These hypothalamic abnormalities result in an increased ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH), ultimately affecting ovarian function in androgen production. The hypothalamus secretes GnRH in a pulsatile manner, likely as a result of chronic progesterone loss associated with persistent anovulation. FSH levels decrease to a point where FSH can no longer support the aromatase activity required to complete follicle development. As a result, LH levels increase but do not reach the LH surge during the middle of the menstrual cycle [13].

A cross-sectional study conducted by Al-Jawadi (2021) indicated that PCOS is associated with a prevalence of gonadotropin dysfunction, characterized by decreased FSH levels and an increased LH: FSH ratio. Our study data showed that vitamin D supplementation in the intervention group increased FSH levels. The LH: FSH ratio was higher in the positive control group compared to the negative control group.

Vitamin D3 supplementation in female rats induced with Testosterone Propionate 1 mg/kgBW (PCOS model) or the positive control group resulted in an increased LH: FSH ratio of 1:2. With the administration of vitamin D3, the treatment group reduced the LH: FSH ratio to 1:4, approaching the normal levels seen in the negative control group. This statement is supported by several studies indicating that vitamin D supplementation therapy has beneficial effects on ovulation function in women with PCOS. Various cellular and molecular mechanisms have been proposed to explain this mechanism [14].

There is a strong indication for further research to directly validate these findings. Further research should include a large sample size and long-term interventions, with double-blind placebo controls. Lastly, the optimal vitamin D dosage should be determined to enable the development of effective therapies.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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