Effect of Phytoestrogens in the Treatment of Polycystic Ovary Syndrome in Rat Model

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Abstract
Polycystic Ovary Syndrome is one of the most commonly occurring endocrine diseases with 5% to 10% prevalence worldwide. Different types of drugs are being used for the treatment of PCOS; however, they have some side effects. This study aimed to investigate the effect of Cinnamon and Flaxseed extract alone and in combination in maintaining the gonadotropins to treat Polycystic Ovary Syndrome and to compare the effect of Cinnamon and Flaxseed extract with standard drug of PCOS (Clomiphene citrate). Estradiol Valerate was used to induce PCOS in rats. 30 female Albino rats were divided into six different groups; control group (received normal saline 0.2 mg), positive control group (0.4 mL of Estradiol Valerate), Clomiphene citrate treated group (20 mg Clomiphene), Cinnamon treated group (40 mg cinnamon extract), Flaxseed treated group (60 mg flaxseed extract) and Cinnamon plus Flaxseed treated group (40 mg cinnamon extract + 60 mg flaxseed extract). The serum concentration of Progesterone, Testosterone, and Estrogen were evaluated in the PCOS-induced rats. After the treatment statistically significant results were observed (p<0.05) serum level of LH was statistically non-significant (p>0.05). Combined therapy treatment showed satisfactory results in restoring the normal hormonal serum levels and the histopathological results showed no cystic follicle in ovaries. Cinnamon plus flaxseed combined therapy treatment improved the serum level of hormones and the histology of ovaries in PCOS induced rats.

Keywords: polycystic ovary syndrome, Estradiol valerate, Clomiphene citrate, FSH, LH, phytoestrogen

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1. Introduction
Polycystic Ovary Syndrome (PCOS) is one of the most commonly occurring endocrine diseases that affects the reproductive aged women and causes structural fluctuations like the formation of cysts. The worldwide prevalence of PCOS is 5-10% [1]. PCOS symptoms which generally occur in patients are: menstrual disturbance, infertility, hirsutism (excessive hair growth), acne and fatness [2] leading to cause obesity, dyslipidemia, insulin resistance (IR), type 2 diabetes mellitus (T2DM), metabolic disorders and coronary diseases [3,4]. Multiple factors are involved in the pathophysiology of PCOS like environmental factors (imbalanced diet, sedentary lifestyle, smoking etc.) genetics, obesity, disturbed hormonal regulation, epigenetic changes during fetal life [5]. Different potential factors may contribute to the presentation and severity of PCOS such as genetics, prenatal insults, epigenetic changes in fetal life. Recently it has been found that genetic factors are also involved in the pathophysiology of PCOS [5]. PCOS diagnosis needs a careful physical examination, laboratory testing and a complete history of the patient [6] (Williams et al., 2016). There are three sets of criteria for the diagnosis of PCOS: The National Institute of Health criteria (1992), Rotterdam criteria (2003) and Androgen Excess Society criteria (2006). These three criteria include anovulation, hyperandrogenism (excess of androgen) and polycystic ovaries for PCOS confirmation [2]. Cinnamon extract containing phytoestrogens have been found to improve the
insulin sensitivity in women with PCOS [7] and regulate the menstrual cycle, decrease the insulin resistance, reduce oligomenorrhea, decrease the level of testosterone and also has the ability to reduce oxidative stress [8].

PCOS treatments are different according to the need i.e., hyperandrogenism, treatment, treatment of menstrual disturbances and treatment against infertility. Drugs used for PCOS treatment are Ethinyl estradiol, Cypotroproterone acetate, Flutamide, Thiaizolidinedione, Metformin, Letrozole, Clomiphene citrate, Insotiol and Statin. For hyperandrogenism, hirsutism and menstrual cycle problems, hormonal contraceptives are used [9].

Currently CC is used as the primary drug for ovulation induction in PCOS patients. Clomiphene is structurally similar to estrogen [9], a selective estrogen receptor regulator with non-steroidal nature having anti-estrogenic ability to deplete estrogen receptors. Treatment of PCOS with CC results in 60 %-80 % increased ovulation but only 20 % conception rate with certain side effects on endometrium and cervical mucus layer and endometrial thickness [10] resulting in inconsistence between the conception and ovulation [11]. Persistent uptake of such type of drugs may induce adverse effects in patients. These side effects are prevented by herbal chemicals and are also used against PCOS [12]. Recently use of phytochemicals has been investigated extensively to treat the PCOS without inducing side effects [10]. Phytoestrogens found in many plants have greater functional and structural similarity to estrogens [13]. Phytoestrogens are linked with the reduction of osteoporosis, cancers, obesity, type 2 diabetes mellitus, cardiovascular problems, metabolic syndrome and hot flashes [14]. The cinnamon extract improves the insulin sensitivity in women with Polycystic Ovary Syndrome [7] and improves the menstrual cycle regulation, decreases the insulin resistance, reduces oligomenorrhea, decreases the level of insulin and testosterone and also has the ability to reduce oxidative stress [8].

Shalaby et al suggested that the use of cinnamon extract improved the antioxidants enzymes activity in diabetic obese rats [15]. The flaxseed plant is an herb scientifically known as Linum usitatissimum. Its extract contains the phytoestrogen, omega-3 fatty acids, alpha linolenic acids and mucilage fibers [7]. Linseed has a rich amount of lignans which are both antioxidants and phytoestrogen and the research on the use of flaxseed or lignan shows reduction in the level of androgen, testosterone [7,16]. Linseed reduces the ovarian size and the number of follicles in polycystic ovaries, maintaining the rhythm of monthlies. Flaxseed reduces testosterone, estrogen, LH and insulin levels and also improves the ovarian function [17].

In this study we wanted to induce the Polycystic Ovary Syndrome by locally available Estradiol Valerate, to check the effect of phytoestrogen containing cinnamon and flaxseed on Polycystic Ovary Syndrome induced rats and compare the standard drug Clomiphene citrate with the phytoestrogens of Cinnamon and Flaxseed against Polycystic Ovary Syndrome in rat models and also compare the gonadotropin levels of all rats before and after the treatment.

2. Materials and Methods

2.1. Ethical Approval and Animals

This study was approved from the Institutional Review Board; The University of Lahore, Approval No: USM/Animal Ethics, Approval/2009/[45,140]. Thirty healthy adult female Albino rats aged 10-12 weeks obtained from the animal house of Institute of Molecular Biology and Biotechnology, The University of Lahore. Before the experiment, the average weight of each rat recorded was 150-200 grams. All the rats were kept in clean cages at room temperature 23°C-25°C with natural light/dark period. The animals had free access to pellet food and water ad libitum. The acclimatization period before the experiment was 2 weeks.

2.2. Estrous Cycle Confirmation

Normal estrous cycle was detected by the vaginal smear method. For the collection of vaginal smear samples, about 0.2 mL to 0.3 mL of normal saline was flushed into the vaginal orifice by using a small dropper. A drop of sample fluid was taken from the vaginal orifice and installed on the glass slide, dried in air, covered with a cover slip and stained with Giemsa stain for microscopic examination. Vaginal smear sample was collected between 8:00 Am to 12:00 Am daily to monitor the regularity of estrous cycle. The rats with a 4 to 5 days’ regular estrous cycle were selected for the induction of PCOS. After the induction of PCOS test was stopped when disturbance in the estrous cycle started, where animals had reached the stage of persistent vaginal cornification that was usually 10 days after the dose of Estradiol Valerate [18].

2.3. Extract Preparation

2.3.1. Cinnamon Extract Preparation

Cinnamon a locally available spice was used for extract preparation. We take 100g of cinnamon and make its fine powder by using grinder. 50g of cinnamon powder was mix in 50 % ethanol in a conical flask, make it airtight by cotton plugs and aluminum foil, kept 30°C temperature on a thermal shaker for 48 hours. After 48 hours, solution was filtered through filter paper, centrifuge at 3000rpm for 5 minutes and the filtered solution was vaporized at normal room temperature in petri plates. After vaporization we obtain pure beneficial cinnamon’s components in powder form. This obtained powder was our cinnamon’s extract used in this experimental study [19].

2.3.2. Flaxseed Extract Preparation

Flaxseeds which are locally available in the market used for extract’s preparation. We take 20 g of flaxseed and soak it in 100 mL of hot distilled water for 30 minutes. After 30 minutes we filtered this flaxseed soaked distilled water to obtain our flaxseed’s beneficial components in a purified form. A filtered slimy solution was obtained that was our flaxseed’s aqueous extract used for this study [20].
2.4. PCOS Induction

PCOS was induced in albino rats by using Estradiol Valerate via hormonal induction method. Estradiol Valerate was purchased from Bayer Pharma (Pvt) Ltd. as a brand name of Gravibinan 2 mL injections for about 15 days. At the 10th day of EV dose, persistent vaginal cornification was observed in rats through the vaginal smear method. PCOS induction was confirmed by the observation of vaginal smear’s stained slides under microscope [21].

2.5. Experimental Design

The animals were divided into six groups including Control group, Positive control group, Clomiphene Citrate treated group, Cinnamon extract treated group, Flaxseed extract treated group and a combined therapy group (Cinnamon extract +Flaxseed extract). Each group consisted of five rats. Group 1: Control group included five rats that received normal saline 1 mL/kg/day orally. Group 2: Positive control group/PCOS group included five rats that were administered with a dose of 2 mL/kg (0.4 mL) of Estradiol Valerate orally through gavage. Group 3: Clomiphene Citrate group included five rats that were administered 100 mg/kg (20 mg) orally through gavage. Group 4: Cinnamon extract group included five rats that were administered 200 mg/kg (40 mg) mixed in 2 mL of normal saline orally through gavage. Group 5: Flaxseed extract group included five rats that were administered 300 mg/kg (60 mg) orally through gavage. Group 6: Cinnamon +Flaxseed extract group included five rats that were administered 40 mg cinnamon extract+60 mg flaxseed extract.

2.6. Biochemical Analysis

2.6.1. Sample Collection

After the completion of the trial, the treated rats were weighed and then anesthetized using chloroform. From each rat, 5 mL of blood was taken from cardiac puncture through 5cc syringes and the blood sample was placed in red topped gel vials/vacutainer. This blood sample was stored in the refrigerator at 4°C before processing.

2.6.2. Serum Separation

After the collection of blood samples from all rats, the blood was allowed to clot at room temperature for about 15-20 min. The blood samples were centrifuged at 3000rpm for 15 min. The serum was collected in Eppendorf tubes and stored at 70°C temperature. Serum was used for the analysis of hormonal assay.

2.7. Evaluation of Ovarian Histology

Ovaries will be removed and settled in 10% formalin solution, enclosed in paraffin and stained by hematoxylin and eosin. Surgical removal of ovaries in all groups will be done for histopathological studies [22].

2.8. Statistical Analysis

The data was expressed in the form of mean and standard error of mean. One-way ANOVA was performed to check out the levels of gonadotropin hormones; LH, FSH, Progesterone, Testosteron and Estrogen in control group, PCOS induced positive control group without any treatment and treatment groups (Clomiphene citrate treated group, Cinnamon extract treated group, Flaxseed extract treated group and Cinnamon plus Flaxseed combined therapy treatment group).

3. Results and Discussion

3.1. Estrous Cycle Detection

Cytology of Estrous cycle was studied and observed in the animals of control group before the induction of PCOS. Estrous cycle was detected by the cytology of vaginal smear. Stained slides were observed under the microscope at 40X. We observed all four phases of estrous cycle and were confirmed by the presence of different types of cells found in these phases. Proestrus phase was detected by the abundance of round shaped nucleated cells found individually or in the form of clusters, Estrus phase detected by the abundance of cornified and anucleated epithelial cells, Metestrus phase was detected by the abundance of cornified and anucleated cells, Diestrus phase was detected by the abundance of leucocytes and Diestrus phase was detected by the abundance of leucocytes that polymorphonuclear in structure (Figure 1). Presence of all such kinds of cells and phases ensures the regularity or normality of Estrous cycle.

3.2. Biochemical Analysis

3.2.1. Luteinizing Hormone

The maximum serum concentration of LH was observed in positive control group after PCOS induction by Estradiol Valerate that maximum serum concentration of LH as compared to the control group. It was followed in descending order by group: cinnamon plus flaxseed combined therapy group, clomiphene group and cinnamon group respectively (Figure 2). The level of LH showed statistically non-significant p value 0.1775.
3.2.2. Follicle Stimulating Hormone

The serum concentration of FSH was significantly lowered in positive control group (p value: 0.0004) after PCOS induction as compared to control group. It was followed in increasing order by groups: Flaxseed, Cinnamon, Clomiphene citrate and Cinnamon Flaxseed combined therapy group after treatment respectively (Figure 3).

3.2.3. Progesterone

The maximum serum concentration of Progesterone hormone was observed in positive control group after PCOS induction as compared to control group. It was followed in descending order by group: Flaxseed extract treated group, Clomiphene treated group and Cinnamon plus Flaxseed combined therapy group after treatment respectively (Figure 4). Serum level of Progesterone showed statistically significant p value: 0.0000.

3.2.4. Testosterone

The maximum serum concentration of Testosterone hormone was observed in positive control group after PCOS induction as compared to control group. It was followed in descending order by group: Flaxseed extract treated group, Clomiphene citrate treated group, Cinnamon extract treated group, Cinnamon plus Flaxseed combined therapy group after treatment respectively (Figure 5). Serum level of Testosterone showed statistically significant variation with 0.0018 p value.

3.2.5. Estrogen

The maximum serum concentration of Estrogen hormone was observed in positive control group after PCOS induction by Estradiol Valerate as compared to the control group. It was followed in descending order by group: Flaxseed extract treated group, Cinnamon plus Flaxseed combined therapy treated group, Clomiphene citrate treated group and Cinnamon extract treated group respectively (Figure 6). Estrogen level showed statistically significant p value 0.0000.

3.3. Histopathological Analysis

For histopathological analysis of ovaries of all control, positive control and treatment group H & E stain was used for staining and the pictures were taken 100X magnification. Histopathological results of dissected ovaries with the fallopian tube of the control group revealed the normal vasculature and ovarian stroma. Normal follicular cysts were present in the ovary,
Graafian follicle and early ovarian follicles were observed and there was no evidence of any kind of malignancy or any inflammatory disease was seen. There was no change in the normal ovarian stroma and vasculature but the ovarian size was enlarged. The size of ovary increased due to the formation of sclerotic multiple cystic follicles and reduced area of corpus luteum which revealed the induction of PCOS. After the administration of Clomiphene citrate histopathological results showed the presence of one cystic follicle and the formation of Graafian follicles in the ovaries of rats. Histology of ovarian tissues after the cinnamon extract treatment revealed the normal ovarian vasculature with the presence of few cystic follicles. The ovary includes one mature Graafian follicle, some secondary follicles. The ovarian vasculature and stroma was normal after the flaxseed extract treatment therapy. There were no cystic follicles in the ovary after the treatment but the formation of early ovarian follicle, secondary follicles were seen. We also see the Graafian follicle. After the combined therapy treatment two mature Graafian follicles were found in ovaries of cinnamon plus flaxseed extract treated group. There was no cystic follicle in the ovaries, secondary follicle was found and corpus luteum returned to its actual volume.

In this research work we wanted to observe the histology of ovaries and the effect of different treatments in the reduction of cystic follicles formed after the induction of PCOS. We found that when we investigated the histology of the EV induced PCOS group we observed six sclerotic polycystic follicles due to EV. Formation of these polycystic follicles linked with hyperandrogenism. For the treatment of PCOS we use a standard drug Clomiphene citrate, cinnamon extract, flaxseed extract singly and in combination. After the treatment we compare the results obtained from Clomiphene citrate treated groups with the results obtained from extract treated groups. We found that cinnamon plus flaxseed combined therapy treatment worked in a promising way by reducing the count of polycystic follicles and formation of mature Graafian follicles.

One of the most commonly occurring endocrine diseases that mainly affects women of reproductive age and causes some structural fluctuations like the formation of cysts [23], was first observed by Stein and Leventhal in 1935 [24]. The worldwide prevalence of PCOS is 5% to 10% [1]. Major symptoms of PCOS are hirsutism, infertility, menstrual disturbance, obesity and acne [2] and all these PCOS symptoms linked with each other i.e. insulin resistance leading to hyperinsulinemia which resulting in adipogenesis and obesity linked with excessive stimulation of LH [25]. Several research studies gave evidence that uncontrolled secretion of gonadotropin releasing hormones from the hypothalamus resulted in the increased secretion of LH [26]. EV treated PCOS rats show androgen excess, irregularity in estrous cycle, anovulation and follicular cysts in ovaries. Such structural changes of ovaries after the single dose of EV are similar to those changes that occur in PCOS patients [1,26]. In this study we use Estradiol Valerate for the induction of PCOS. Thirty female Albino rats were divided into six different groups; 1) Control group, 2) Positive control group, 3) Clomiphene citrate treated group, 4) Cinnamon treated group, 5) Flaxseed treated group and 6) Cinnamon plus Flaxseed combined therapy treatment group. Estrous cycle of rats lasts for 4-5 days and has 4 different phases: Proestrus, Estrus, Metestruis and Diestruis. In a previous study for the detection of estrous cycle, the smear collected from the vagina of rats contains three different types of cells: leukocyte, nucleated and cornified epithelial cells. Four stages of the estrous cycle based on the proportion of all these three types of cells. Proestrus characterized with the presence of nucleated, round shaped cells found individually or in the form of clusters with few anucleated cells, estrous phase characterized by the abundance of cornified anucleated epithelial cell, metestruis characterized by the abundance of leukocytes and diestrous phase characterized by the abundance of leukocytes that are polymorphonuclear [27]. In this present study we found all types of cells that confirm all four phases of the estrous cycle. We observed nucleated cells in round shape that detect the Proestrus phase of estrous cycle, cornified anucleated epithelial cells in abundance detect the estrus phase, abundance of leukocytes detects the Metestrus phase while leukocytes with polymorphonuclear structure detect the Diestruis phase of estrous cycle.

In this research work we found the increased serum level of LH, progesterone, testosterone and also the elevated level of estrogen in positive control group as compared to control group but FSH showed decreased serum concentration in positive control group as compared to control group. The results obtained after the induction of PCOS were supported by previous research works. Previous studies discussed that women with this endocrine disorder showed an increased level of E2, testosterone and LH but decreased in FSH. This LH increased concentration leading to the stimulation of androgen and an increase in the PCOS patients [28] (Naseri et al., 2020). Osman et al., 2019, discussed the PCOS induction by Estradiol Valerate and observed an elevated level of FSH, LH and testosterone but progesterone level decreases with no significant change in E2 level. Elevated levels of LH trigger the excessive production of androgen which in turn leads to hyperandrogenism and also develop the PCOS reported by Bayrami et al., 2020. This increased serum level of LH leading to the excessive production of Progesterone. Reduced serum concentration of FSH leading to anovulation resulting in the development of PCOS, stated by Zeba et al., 2018. Clomiphene citrate is used as the primary drug for ovulation induction in PCOS patients. Clomiphene is structurally similar to estrogen (Atashpour et al., 2017), a selective estrogen receptor regulator with non-steroidal nature having anti-estrogenic ability to deplete estrogen receptors (Atashpour et al., 2017). Xiong et al., 2018 observed the serum concentration of sex hormones after the combined therapy of Clomiphene citrate and Metformin in PCOS patients. He observed the elevated level of FSH and Estradiol (estrogen) but reduced concentration of LH. Serum level of FSH and progesterone were increased while the level of LH and estrogen was decreased (Atashpour et al., 2017).
These studies supported the results of our present research work. We investigate the serum levels of reproductive hormones in PCOS induced rats after Clomiphene citrate treatment therapy and we observed that there was a significant reduction in LH serum level as compared to the positive control group, progesterone, testosterone and estrogen serum level in Clomiphene citrate treated rats but the level of FSH increased as compared to positive control group. Long-term uptake of such types of drugs may induce adverse effects in patients, for the inhibition of these side effects researchers and scientists suggest herbal treatments (Atashpour et al., 2017). Phytoestrogens found in many plants have greater functional and structural similarity to estrogens (Gaffer et al., 2018) and have the ability to bind with estrogen receptors. In this experimental research work we use phytoestrogen-containing foods; Cinnamon, Flaxseed and also used both of them as a combined therapy treatment against PCOS. We observed the hormonal levels of sex hormones in EV induced PCOS rat after Cinnamon extract treatment. Cinnamon improves the hormonal imbalance by restoring their significant serum level. Cinnamon increases the concentration of FSH as compared to the positive control group but reduced serum level of LH, progesterone, testosterone and estrogen were observed in that group. Duo et al., 2018, also observed the same elevated levels of LH and testosterone in Cinnamon treated mouse as compared to PCOS mouse but Khodaeifar et al., 2019, investigated the lowered concentration of FSH, LH, testosterone but higher concentration of estrogen as compared to PCOS induced groups. A group of PCOS induced rats was used to investigate the effect of Flaxseed extract on the hormonal level of these rats and we observed the decreasing trend of all hormone; LH, FSH, testosterone, progesterone and estrogen hormone in treated groups as compared to positive control group. Osman et al., 2019, observed and discussed the decreased concentration of FSH, LH and testosterone but increased hormonal level of progesterone with no significant effect on estrogen level. Jelodar et al., 2017, also discussed the elevated hormonal level of testosterone with no change in estrogen level. A study on the combined therapy of Flaxseed and Spearmint for PCOS treatment showed the elevated concentration of testosterone and progesterone but decreased concentration of estradiol discussed by Mehraban et al., 2020. In this study we use a combined therapy of Cinnamon extract with Flaxseed extract for PCOS induced female rats to find more beneficial and significant results. We observed the elevated level of FSH as compared to positive control.
group while the lesser concentration of LH, progesterone, testosterone and estrogen in combined therapy treatment group as compared to the positive control group. There was no cystic follicle in the ovaries. Histopathological results of dissected ovaries with the fallopian tube of the control group revealed the normal vasculature and ovarian stroma. Four follicular cysts were present in the ovary and there was no evidence of any kind of malignancy or any inflammatory disease was seen. We found no change in the normal ovarian stroma and vasculature but the ovarian size was enlarged. The size of ovary increased due to the formation of six sclerotic multiple cystic follicles which revealed the induction of PCOS while there was no evidence of inflammatory disease or any type of malignancy. These results coincide with previous research work of Amini. Amini et al., 2016, reported that when PCOS was induced by EV, normal preovulatory follicles were reduced in number and the percentage of corpus luteum was also decreased. After the administration of Clomiphene citrate histopathological results showed the presence of one cystic follicle and the formation of three graafian follicles in the ovaries of rats. Similar results have been observed by Lombardi et al., 2020, who observed the increased count of ovarian follicles but cystic follicles were reduced in number. Khodaeifar et al., 2019, investigated the Cinnamon treated animals and observed that cystic follicles decreased in number after the treatment while normal ovarian follicles were formed. These results are similar to the results of our research work. Histology of ovarian tissues after the cinnamon extract treatment revealed the normal ovarian vasculature with the presence of few cystic follicles. The ovary includes one mature graafian follicle. The ovarian vasculature and stroma was normal after the flaxseed extract treatment therapy. There were no cystic follicles in the ovary after the treatment but the formation of ovarian follicles was seen. Histological results of our experimental research work supported by the research work of Mehraban et al., 2020 who found the reduction in the formation of cystic follicles. After the cinnamon plus flaxseed combined therapy treatment two mature graafian follicles were found in ovaries of cinnamon plus flaxseed extract treated group.

Results of biochemical analysis and the histopathological analysis revealed that the cinnamon plus flaxseed combined therapy treatment was more effective in comparison to Clomiphene citrate treatment, cinnamon extract treatment and flaxseed extract treatment. Cinnamon plus flaxseed combined therapy treatment showed promising effects in restoring the hormonal serum concentration of LH, FSH, progesterone, testosterone and estrogen and also reduced the amount of multiple cystic follicles after the treatment.

5. Conclusion

The possible cure of Polycystic Ovary Syndrome may lie in the use of plant extracts i.e., Cinnamon and Flaxseed having phytoestrogens. From the literature it is also found that their use helps in maintaining the gonadotropin levels in the treatment of PCOS. So we use these phytoestrogens containing plants (Cinnamon and Flaxseed) against PCOS. We compare the levels of female reproductive hormones before and after the treatment of Estradiol Valerate induced PCOS rats and also compared these phytoestrogens containing plant extracts with the standard drug Clomiphene citrate which is used extensively for the treatment of anovulation and infertility in PCOS patients. We conclude that plant extracts are better than drugs in maintaining and restoring the hormonal balance and also restore the histological changes which occurred after PCOS induction and the cinnamon plus flaxseed extract combined therapy treatment work significantly against PCOS in female Albino rats.

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