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Investigation of the relationship between prolactin and infertility by expression levels of kisspeptin (KISS1), KISS1 receptor, neurokinin (NK), NK receptor genes

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ABSTRACT

Objectives: Prolactin level, which rises in the blood during pregnancy and after birth, plays a role in physiological events such as metabolism and reproduction. Increased blood levels of prolactin lead to various disorders. The most important of these are amenorrhea/galactorrhea and disruption of ovulation. Disruption of ovulation is a serious problem and can lead to infertility problems. Kisspeptin (KISS1) and Neurokinins are involved in the control of ovulation. This study aimed to explain the relationship between Prolactin and infertility with the expression levels of KISS1, KISS1 Receptor (KISS1R), Neurokinin (NK), Neurokinin Receptor (NKR) genes.

Methods: Fourty female Wistar Albino rats, 12-16 weeks old, were used in this study. Three groups were formed, 10 rats were in Group-1 as Control, 10 rats were in Group-2 given Saline, and 20 rats were in Group-3 given Metoclopramide (Metpamid, Sifar Turkey). The raising effect of Metoclopramide on blood Prolactin levels leading to infertility was helpful to clarify the process in comparison with the other two groups not given Metoclopramide.

Results: In Group-3 given Metoclopramide, the blood Prolactin levels were found to be significantly higher compared to Group-1 and Group-2 subjects that were not given this drug. Expression values of KISS1, KISS1R and NK, NKR genes were found to be significantly decreased in Group-3, where the Prolactin level increased, compared to the other two groups ($P<0.001$).

Conclusions: In this study, it has been shown that the expression levels of Neuropeptide genes (KISS1, NK) are suppressed with the direct effect of hyperprolactinemia, thus decreasing the amount and functions of Neuropeptides. In our study it was concluded that the negative effects of prolactin elevation on reproduction may be mediated by neuropeptides.

Keywords: Fertilization, infertility, kisspeptin, neuropeptide, prolactin, reproduction

Infertility has been defined by the World Health Organization as a serious universal problem. It is estimated that approximately 17.5% of reproductive-aged couples worldwide has infertility or subfertility [1]. In most cases of infertility, there are problems in the release of hormones that govern the reproductive system. Hyperpolactinemia has an important place among these problems and this condition is more

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common in women. While its prevalence is 0.4% in the adult population, it can be as high as 9-17% in women with reproductive disorders [2]. These values show the effect of Prolactin on infertility, and high Prolactin exerts its inhibitory effect on the hypothalamus [3]. It is known that there is gonadal dysfunction in the case of hyperpolactinemia. LH (Luteinizing Hormone) peak cannot occur in the anovulatory cycle or ovulation is prevented because the progesterone phase is blocked. Gonadotropin Releasing Hormone (GnRH) dependent LH and Follicle Stimulating Hormone (FSH) releases are triggered by Kisspeptins. This has been demonstrated in mice and rats with mutations of G protein coupled receptor 54 (GPR54). Peripherally administered Metastin (Kisspeptin) increases gonadotropin levels in female and male rats and causes ovulation in prepubertal females [4, 5]. Kisspeptins are members of the RF amide-related proteins family and are important regulators of the GnRH neuroendocrine system [6].They are Neuropeptides that act on GPR54 receptors by deriving from a 145 amino acid precursor protein, which is located in the q32 region of chromosome 1 and is transcribed by the gene called kisspeptin (KISS1) [7, 8]. Kisspeptin neurons play a role as the primary mediator in the feedback control of GnRH release [9, 10]. Another protein found to be associated with the release of GnRH in the hypothalamus is Neurokinin B (NKB). This signal peptide is a member of the Tachykinin family and is involved in intracellular signaling in vertebrates and invertebrates. The most well-known Tachykinins in man are Substance P (SP), Neurokinin A (NKA), and NKB, all of which are commonly synthesized in the central nervous system. NKB and its receptor NK3R, which play a role in the human reproductive axis, are encoded by Tachykinin 3 (TAC3) and Tachykinin 3 receptor (TACR3) genes, respectively. Their mouse homologues are the Tachykinin 2 (TAC2) and Tachykinin 2 receptor (TACR2) genes. These genes are widely expressed in the central nervous system of humans and rodents. The expression of NKB and Kiss1 in the same cells in the arcuate nucleus indicates that they may be closely related. Indeed, studies on mice have shown that NKB agonist increases KISS1 expression and thus affects GnRH and subsequently LH release [10, 11]. It has been stated that KISS1 and NKB may be related to each other, as well as to other hormones that have an effect on GnRH secretion in the living system, especially in the hypothalamus. One of them is Prolactin. Hyperprolactinemia prevents fertilization by inhibiting ovulation with its inhibition effect on GnRH secretion. It was aimed to eliminate the inhibition effect of Prolactin on GnRH with the application of Kisspeptin1 to rats with hyperpolactinemia. Thus, anovulation improved and ovulation occurred with the positive effect of KISS1 on GnRH [12].Considering all this literature information, in this study, we examined the related Neuropeptides we identified and the expression levels of their genes. Hyperprolactinemia reduces the expression levels of KISS1 and Neurokinins, called as Neuropeptides, and their receptors genes, thereby stopping their effects that increase GnRH secretion. Thus, high Prolactin level in the blood reduces the functions of these special Neuropeptides and their receptors, and inhibits the release of GnRH through them. In other words, hyperprolactinemia triggers this metabolic process, makes the related Neuropeptides and their receptors hypoactive, indirectly inhibits the release of GnRH upon this hypoactivation, thus providing infertility.

METHODS

Animal Model

In this study, we created a female animal model that their reproduction abilities were inhibited through the high blood Prolactin level raised with Metoclopramide. Once the Metoclopramide administration was stopped, reproduction was possible in the same female rats again, when the blood Prolactin level returned to normal. This experimental animal model enabled to observe the increase of blood Prolactin in favor of infertility and also its decrease in favor of fertility. This exemplary model provided an important knowledge base for the investigation of all infertility problems, including the disturbed menstrual cycle, in determining the negative side effects of Metoclopramide-containing drugs, which have been applied clinically for various treatments in humans for years.

Three groups of Wistar Albino female rats weighing 200-250 g selected as 12-16 weeks old were used in this study. The number of rats was 40 in total. They were divided into three groups, including 10 for Controlgroup,10 only Saline administered group, and 20 only Metoclopramide (Metpamid, Sifar Turkey) ad-

ministered group, so that we separated them as Group-1-2-3 consecutively, as shown in Table 1. Blood Prolactin levels and expression levels of KISS1, KISS1 receptor (KISS1R), Neurokinin (NK) and Neurokinin receptor (NKR) genes of all these rats were examined. Table 1 summarizes all these three groups of female rats and their applications.

It was determined that fertilization was inhibited when Metoclopramide was given to female rats, and fertilization was reactivated when Metoclopramide was stopped. Thus, Group-3 was given Metoclopramide for two weeks, and then blood Prolactin levels of rats in all groups were measured. The values measured using the Prolactin ELISA immunoassay kit were compared. It was observed that blood Prolactin levels increased significantly in rats in Group-3, and there was no change of blood Prolactin levels in rats Group-1 and Group-2. Meanwhile, rats in Groups-1, Group-2, and 10 randomly selected rats from Group-3 were additionally used in mRNA Expression studies. Vivantis RNA isolation kit (Vivantis Tecnologies Sdn Bhd) was used for RNA isolation. Using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™), cDNA was obtained from RNAs. In our study, we performed Real-Time (RT) PCR reaction using TaqMan Gene Expression Assay containing FAM stained probe designed for Kiss1, Kiss1 receptor, Neurokinin and Neurokinin receptor genes. Differences between Threshold cycle (Tc) and Crossing points (Cp) values were measured to determine expression levels.

Ethical Permission

The use of experimental animals in this study was allowed and recorded with reference decision number 1 approved in the 8th session of the local ethics academic authority named Cukurova University Medical Faculty Experimental Research and Application Center dated 21August 2013. The animals were carefully treated in accordance with the guidelines confirmed by this official permission.

Statistical Analysis

When summarizing the data, variables are expressed as mean ± standard deviation median (min-max) value. The Kruskal-Wallis Test was used for three or more group comparisons, and then the Mann-Whitney U test was used for pairwise comparisons or direct two group comparisons for expression values. In the evaluations of the variables, differences where $p<0.05$ were considered statistically significant. IBM SPSS Statistics 28.0.1 package program was used in the analysis of the data. Prolactin levels in Group-3 given Metoclopramide were found to be significantly higher than Group-1 and Group-2 who were not given this drug. Expression values of Kiss1, Kiss1 receptor and Neurokinin, Neurokinin receptor genes were found to be significantly decreased in Group-3, where the Prolactin level increased, compared to the other two groups ($P<0.001$). Prolactin levels were measured in these three groups. As a result of the analysis, Prolactin level was the highest in Group-3 given Metoclopramide, Prolactin levels were found to be low in Group-1 and Group-2, which were not given Metoclopramide.

Differences between Threshold Cycle (Ct) and Crossing Points (Cp) values were measured to determine expression levels. The Gene Expression levels of the subjects in the Control group were calculated with the mRNA Gene Expression Assay for comparisons with the other groups.

Table 1. Group of experimental animals (rats)

Groups of rats	Saline application	Metoclopramide application	Expression evaluation	Follow up of pregnancy
Group-1	-	-	+	+
Group-2	+	-	+	+
Group-3	-	+	+/-*	+/-*

*10 randomly selected female rats from Group-3 were combined with male rats for fertilization after the experimental applications related to all these groups.

The differences between the rats in Group-3 given Metoclopramide and the other groups were found to be statistically significant ($P<0.001$), as shown in Table 2.

RESULTS

It was determined that fertilization was inhibited when Metoclopramide was given to female rats, and fertilization was reactivated when Metoclopramide was stopped. Thus, Group-3 was given Metoclopramide for two weeks, and then blood Prolactin levels of rats in all groups were measured.

Prolactin levels were measured in three groups. As a result of the analysis, Prolactin level was the highest in Group-3 given Metoclopramide, Prolactin levels were found to below in Group-1and Group-2, which were not given Metoclopramide (Table 2).

Expression values of KISS1, KISS1R and NK genes were found to be significantly decreased in Group-3, where the Prolactin level increased, compared to the other two groups (P<0.001) (Figs.1, 2, and 3).

No statistically significant difference was found in Group 3 in terms of NKR expression value (P=0.052) (Fig. 4).

Following the experimental applications,10 randomly selected female rats from Group-3 were brought together with male rats and their pregnancy status was examined. It was determined that 8 of the rats became pregnant after 3 months, and 2 of them became pregnant after 4 months.

DISCUSSION

Infertility is defined as the inability to achieve pregnancy within 12 months despite regular vaginal sexual intercourse with the same partner without using any contraceptive method [1]. Infertility can be caused by various reasons. Prolactin is one of the hormones associated with infertility. The idea that high blood Prolactin levels can lead to infertility by decreasing GnRH secretion is an important fieldof research [13]. In studies conducted to show the relationship between Prolactin and infertility, blood Prolactin levels of the subjects were increased and fertilization decreased [12]. It is noteworthy that in another study, all transgenic female mice unable to synthesize Prolactin were found to be infertile [14]. In this case, if the level of Prolactin in the blood is zero, it is an important issue that should be investigated further. This may be a different metabolic process to compensate for the absence of Prolactin and perhaps gains more activity than necessary, which can also cause infertility. By the way, Ormandy *et al* detected many reproductive disorders such as disruption of the menstrual cycle and decreased fertilization in transgenic mice with mutated Prolactin receptor gene [15]. In our study, blood Prolactin levels of the Metoclopramide group were found to be significantly higher than the Control group and the Saline group. Owing to our experimental animal model, we succeeded to realize Prolactin elevation as endogenous and reversible. In this way, we observed that female rats given Metoclopramide did not become pregnant, but pregnancy occurred in the same rats after the effect of Metoclopramide was over. We noticed that pregnancies were absolutely normal in female rats in Group-1 and Group-2, which were not given Metoclopramide. All 10 randomly selected female rats from Group-3 gave birth 3 months after Metoclopramide administration was stopped. This development, which

Table 2. Distribution of prolactin levels and comparisons between the groups of rats

Groups of rats	Prolactin levels (ng/mL)	P value (multiple comparisons)
Group-1 (Control)	23.0±5.4 20.9(18.5-35.7)	**-**
Group-2 (Saline)	29.7±10.0 28.5(19.7-55.9)	**0.364***
Group-3 (Metoclopramide)	53.3±14.3 53.0(34.0-73.4)	**<0.001*** **0.017****
P value	**<0.001**	

Data are shown as mean±standard deviation and median (minimum-maximum) *Comparison with Control, **Comparison of the other two groups

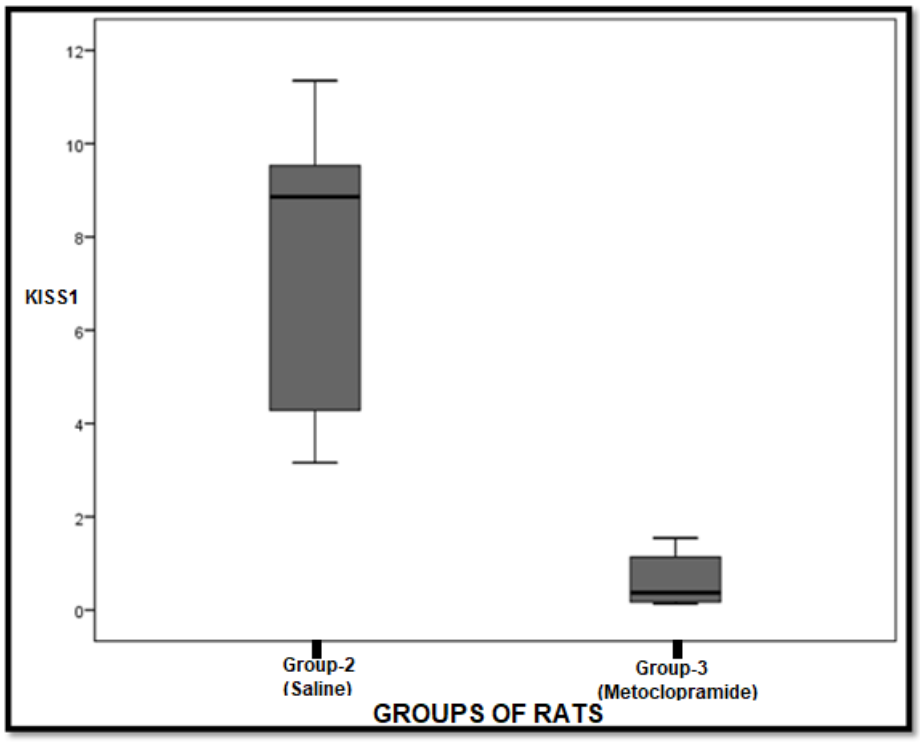

Fig. 1. Expression levels of KISS1 in saline and metoclopramide Groups. The difference in expression levels of KISS1 between Group-2 (Saline) and Group-3 (Metoclopramide) was statistically significant (P<0.001).

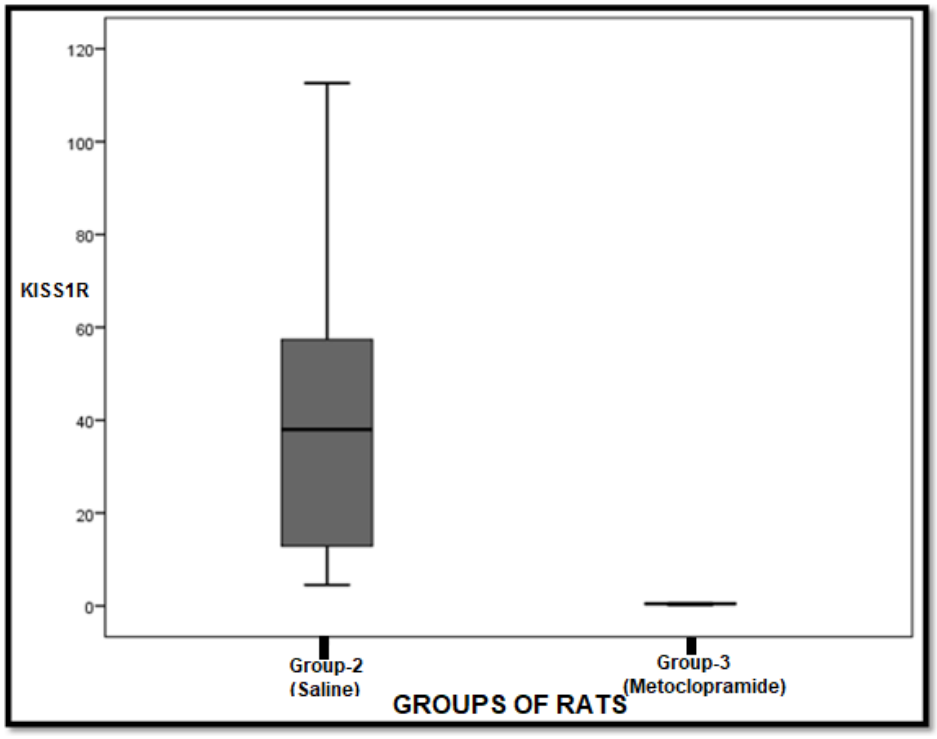

Fig. 2. Expression levels of KISS1R in saline and metoclopramide groups. The difference in expression levels of KISS1R between Group-2 (saline) and Group-3 (metoclopramide) was statistically significant (P<0.001).).

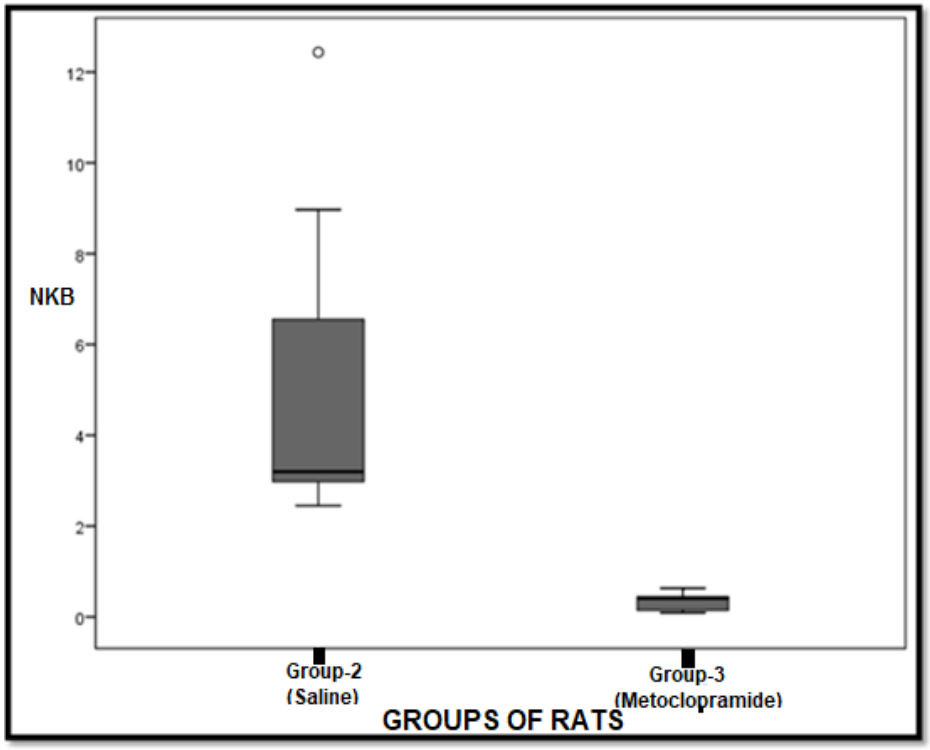

Fig. 3. Expression levels of NKB in saline and metoclopramide Groups. The difference in expression levels of NKB between Group-2 (saline) and Group-3 (metoclopramide) was statistically significant (P<0.001).

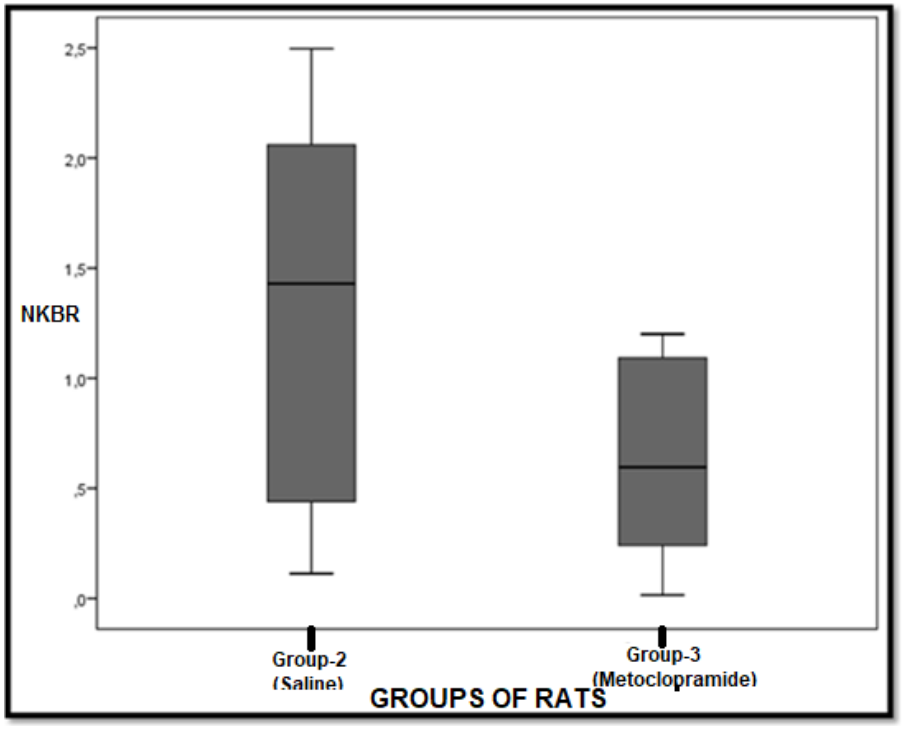

Fig. 4. Expression levels of NKBR in saline and metoclopramide groups. The difference in expression levels of NKBR between Group-2 (saline) andGroup-3 (metoclopramide) was considered tobe borderline significant (P=0.052).

resulted in fertilization and delivery, confirms our opinion about the effect of Metoclopramide, which triggers infertility by elevating the Prolactin level in the blood. If the effect of Metoclopramide is over, the inhibiting pressure of Prolactin on ovulation will be diminished, because Prolactin blood level will decrease to normal gradually after stopping the experimental Metoclopramide administration. Bachelot and Binart suggested that Prolactin causes anovulation by inhibiting the release of GnRH and thus decreasing the amount of LH and FSH in the blood. However, they did not elucidate how Prolactin does this inhibition and the existence of Neuropeptides that mediate it [16].The results of our study enabled use to set an example that can lead to new research on this subject. Meanwhile, several other studies have provided new evidence that Prolactin may modulate the reproductive axis by acting on a specific population of hypothalamic neurons expressing the KISS1gene [17,18].

Loss-of-function mutations in genes encoding Kisspeptins or in the KISS1R cause puberty problems and infertility by negatively affecting the reproductive system in humans and animal models [19]. In the study of Topaloğlu *et al*. [20], it was shown that hypogonadotropic hypogonadism developed in all subjects with KISS1 mutation. In another study, the suppressive effect on GnRH caused by high blood Prolactin levels was abolished with KISS1 injection, thereby returning the ovulation process to normal. The fact that ovulation occurs again by daily injections of KISS1 to female mice that become infertile with Prolactin infusion confirms our comments [12]. In our study, we showed that elevated Prolactin decreased KISS1 and KISS1R expressions in female rats by comparing with our Control and Saline groups. KISS1, which has proven to play an important role in increasing GnRH secretion, is not the only Neuropeptide to undertake this task. Studies conducted in recent years have shown that NKB also has effects that increase the release of GnRH [21]. The neuroanatomical relationship between Kisspeptins and NKB and the demonstration that both are effective in GnRH stimulation and gonadotropin release suggest that these two Neuropeptides may mediate the effects of each other on puberty and fertility [22]. The data we obtained in our study also supports this thesis. Expression of NBP was significantly decreased in Metoclopramide administered subjects compared to Control and Saline groups.

CONCLUSION

This study revealed that KISS1, KISS1R, NK and NKR mRNA expressions were significantly decreased in our subjects whose blood Prolactin levels were elevated by using Metoclopramide for comparison to the Control and Saline groups. All data are compatible with the literature and give an accurate idea to explain the inhibitory effect of Prolactin on the HPG axis. In light of these results, many kinds of pharmacological agents can be checked out, if they have any effect of raising blood Prolactin levels so that their biological effects can lead to this specific clinical outcome of infertility. As a matter of fact, the present literature in this field is not enough to fully understand the whole cycle. For this reason, GnRH neuron activities, histopathological evaluation of the hypothalamic region, receptor functions in the uterus, and many more factors should also be examined in detail.

Authors' Contribution

Study Conception: EA; Study Design: MBY, EA; Supervision: MBY, EA; Funding: EA; Materials: EA; Data Collection and/or Processing: EA; Statistical Analysis and/or Data Interpretation: EA, MBY; Literature Review: EA; Manuscript Preparation: EAand Critical Review: EA.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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