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Histone Methylation in the CYP11A1 Gene Promoter in the Follicular Granulosa Cells Extracted from the Women Referring to theFertility Treatment Clinic in Tabriz, Iran

Saad Ghaderi[1], Masoud Maleki [*2]

1- Department of biology, Islamic Azad University, Tabriz Branch, Tabriz, Iran.
2- Department of biology, Islamic Azad University, Tabriz Branch, Tabriz, Iran.

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Correspondence:

Masoud Maleki

Azad University, Tabriz branch, Tabriz, Iran

Maleki.masoud@gmail.com

Abstract

Infertility is defined as the absence of pregnant after one year of regular unprotected sexual intercourse. In women, infertility may be caused by several factors, all of which may be affected by genetic factors. Epigenetic changes play a key role in embryonic development, ovulation, and spermatogenesis. During oocyte growth, follicular cells form a multilayer coating of granulosa cells, which are affected by gonadotropin hormones and secrete steroid hormones. TheCYP11A1gene is also involved in the pathway of steroid hormone production in luteinizing granulosa cells. The CYP11A1 enzyme converts cholesterol into pregnenolone in the progesterone production pathway. Progesterone is an important steroid hormone, which is significantly involved in fertility and pregnancy. Histone modifications also contribute to the induction of the CYP11A1gene, and methylation of histone H3 on lysine 4 (H3K4me) in the regulatory region of the gene leads to the increased expression and activation of gene transcription. The present study aimed to evaluate the methylation level of H3K4me3 in the CYP11A1 gene regulatory region in the granulosa cells extracted from the fertile and infertile women referring to the infertility treatment center in Tabriz, Iran. Chromatin immunoprecipitation (ChIP) and real-time polymerase chain reaction were used to evaluate the methylation level. According to the results, the fertile and infertile women had no significant difference in terms of the H3K4me3 methylation level at the CYP11A1 gene regulatory region. In addition, no significant correlations were observed between histone methylation at the CYP11A1 gene promoter region, follicle rate, and egg maturation.

Keywords: Granulosa Cells, Histone Methylation, Steroidogenesis, CYP11A1 Gene

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Introduction

Infertility is among the most traumatic experiences in life, which could become a crisis within social and psychological contexts. In medical terms, infertility is whena couple fails to become pregnant after one year of regular unprotected sexual intercourse. Infertility is mainly classified as primary and secondary (1).

In women, infertility may be caused by several factors, such as ovulation problems (1), age-related problems (chance of fertility decline accelerates since the age of 35-40 years and reaches almost zero at the age of 45 years) (2), early ovarian failure (incompatibility of sex cells causes ovarian failure) (3), polycystic ovary syndrome (large ovaries with small cysts) (4), endometriosis (estrogen-dependent inflammatory disease caused by the presence of the endometrial tissue outside the uterine cavity) (5), and chromosomal diseases (e.g., Turner syndrome and Klinefelter syndrome in men) (6). According to the literature, the prevalence of infertility has been on the rise recently. Therefore, scientific research and epidemiological studies are essential to determining the etiology of infertility in men and women.

Epigenetic mechanisms play a pivotal role in the activation or deactivation of the genes that are effective in egg development, embryo formation, and embryonic development, and these processes have significant effects on fertility/infertility; therefore, extensive research in this regard is of paramount importance. Some findings have provided solutions for the effective treatment of infertile women, and the success of assisted reproductive technology (ART) has been confirmed in this regard.

Hormones are another influential factor in infertility in women, and the function of the genes that are involved in hormone secretion (especially steroid hormones) must be thoroughly investigated. Furthermore, epigenetic changes play a key role in the steroidogenic pathway, and histone modifications affect the activation or inhibition of gene transcription in this pathway; therefore, adequate research must be focused on these changes, especially since the methylation of steroidogenic genes in infertile women and its role as a result of ART in women in Iran have not been properly investigated.

Histone modifications are the key regulators of gene expression in many diseases, including infertility. However, data is scarce regarding the histone changes in the CYP11A1gene regulatory region in infertility.

The present study aimed to evaluate H3K4me3 histone methylation in the CYP11A1 gene promoter in the granulosa cells of infertile and fertile women with children and compare their histone methylation in terms of follicle number and egg quality.H3K4me3 histone methylation in the CYP11A1 gene promoter was the independent variable, and the number of follicles and number and quality of eggs were the dependent variables.

Literature Review

In a study, Lingu et al. reported changes in H3K4me3 at the H4K8, H4K5, H4K16, and H4K12sites, as well as three histones in the H3K14 and H3K9sites(7). Moreover, Kageyama et al. stated that DNA methylation remained constant in the primordial to the early follicles, while gradually increasing in the form of the antrum. In the mentioned

study, the increase in the methylation of mice startedon days 10-15 (8).

In another research, Hyura et al. reported that DNA methylation depended on the oocyte size. In addition, DNA methylation mostly occurredin the oocytes with the diameters of 55-60 microns (9).According to Silvia et al., histone acetylation declined slightly during the early stages of oocyte growth, followed by an abrupt increase during follicle development (10).

In a researchperformed on birds (2001), the qPCR studies indicated that the frequency of the CYP11A1gene transcription changed in the ovary, oviduct, and pituitary in the different stages of the reproductive cycle. In addition, it was reported that increased or decreased DNA methylation affected the expression level of important steroidogenic genes in follicular cells (11). In another research conducted in 2015, ovarian follicles were reported to be able to synthesize estrogen and progesterone, which are essential to egg development. Moreover, the qPCR analysis demonstrated that theStAR and CYP11A1genes are differentially expressed in follicles in various stages, and their expression may increaseor decrease by DNA methylation.

In another study, Norinio et al. (2001) evaluated the DNA methylation and histone modifications of the StAR andCYP11A1genes after the injection of human gonadotropin (hCG) using the chromatin immunoprecipitation(ChIP) technique. The obtained results were indicative of the impact of the epigenetic in the promoter region on the increased CYP11A1 gene expression after luteinizing hormone (LH) increase (12).

Materials and Methods

The ChIP technique isused to evaluate the level of the epigenetic factors in the CYP11A1 gene regulatory region. In this method, the interaction between a particular protein and specific regions of the genome is examined, and detection is performed using the real-time polymerase chain reaction (RT-PCR) method. Therefore, we applied the ChIPand RT-PCR techniques. In the present study, the H3K4me3 histone modifications in the CYP11A1 gene regulatory region were evaluated using theChIP technique in four stages, including the stabilization of the cells and developing connections between protein and chromatin, celllysis and chromatin shearing, the immunoprecipitation of the cross-linked chromatin, and DNA purification.

The primer was designed from the promoter region of the CYP11A1 gene in order to evaluate the epigenetic changes in the CYP11A1 gene regulatory region, followed by the use of RT-PCR. In addition, the IP samples containing the chromatic regions were attached to the H3K4me3 epigenetic marker antibodies. In contrast, the input samples contained all the chopped chromatin pieces, and the IP and Input samples were eventually compared.

Results

4.1. Evaluation of the Level of Epigenetic Factors in the CYP11A1Gene Regulatory Region Using the ChIP Technique.

Data analysis was performed in SPSS using various statistical tests, and diagrams were illustrated and presented. In order to estimate the presence of H3K4me3 methylation and

measure the effect of chromatin deposition on immunity, we used the data obtained from the RT-PCR method based on the following equation to achieve the input rate:

%INPUT = AE^ (Ct input- Ct ip)Fd ×100

In the equation above, AEisthe efficiency of replication, which is equal to two in ideal conditions? In the current research, the efficiency of replication was estimated to be less than 1.98 based on serial dilution experiments of the primers and following equation:

AE= 10^ (-1/ slope)

In the equation above, the dilution compensatory factor (Fd) is to compensate for the difference in the IP and input DNA level, and since the input samples were 10% of IP, Fd was calculated to be 0.1.

Figure 1. Level of H3K4me3 in Infertile Women

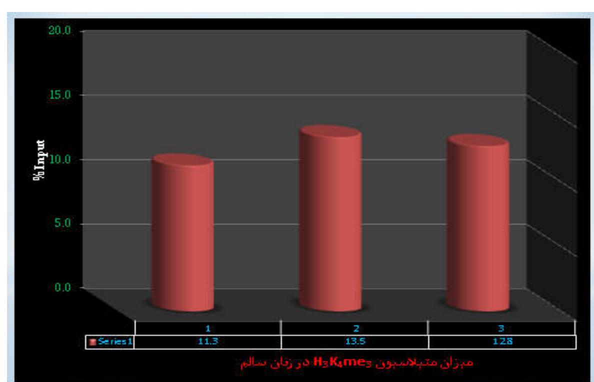

Figure 2. Level of H3K4me3 in Healthy Women

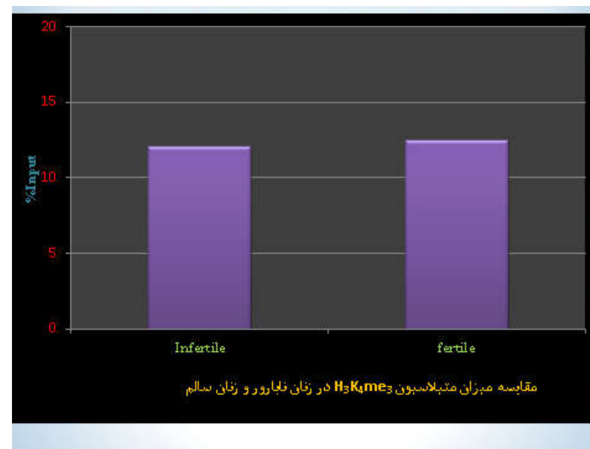

Figure 3. Comparison of Fertile and Infertile Women in Terms of H3K4me3

4.2. Statistical Analysis

The medical files of the subjects were reviewed to determine the correlations between the CYP11A1gene methylation level, number of follicles, and egg quality at theGV, M1, and M2 stages. In addition, H3K4me3 in the CYP11A1 gene regulatory region was assessed in the fertile and infertile women using the ChIP technique, and the samples were evaluated in terms of the number of follicles and egg quality at the GV, M1, and M2 stages of the cellular cycle. The ChIP results were analyzed using RT-PCR.

4.2.1. Descriptive Statistics

The statistical indices in the current research were mean, standard deviation, frequency, minimum, and maximum. In addition, the distribution of the studied variables was expressed using frequency tables and bar charts.

4.2.1.1. Specificity of the Respondents in Terms of Infertility Type

Table 1. Frequency Distribution of Subjects Based on Type of Infertility

Type of Infertility	Frequency	Percentage
Primary	12	66.6
Secondary	3	16.7
Healthy (fertile)	3	16.7
Total	18	100

Table 2. Descriptive Statistics of Quantitative Research Variables

Variable	F	Mean ± SD	Min- Max
Follicle Rate	18	24.22 ±11.97	6 - 54
Egg Rate at GV Stage	11	4.36 ±3.44	1 - 10
Egg Rate at M1 Stage	15	2.60 ±1.64	1- 7
Egg Rate at M2Stage	18	17.17 ±9.90	4 - 46
$CYP_{11}A_{1}$Gene Methylation Rate	18	12.19 ±1.87	9.50-16.20

According to the information in Table 2, the mean follicle and egg rates at the GV, M1, and M2 stages and CYP11A1 gene methylation ratewere estimated at 24.22±11.97, 4.36±3.44, 2.60±1.64, 17.17±9.90, 12.19±1.80, and 1.87, respectively. It is notable that among 18 subjects, the egg rate was not recorded in seven and three subjects at the GV and M1 stages, respectively.

4.2.2. Normal Distribution of the Variables

In the present study, the assumption in most of the statistical tests was that the studied variables had normal distribution. The Kolmogorov-Smirnov test was used to determine whether the distribution of the quantitative variables had normal distribution, and the null and alternative hypotheses of the test were as follows:

H0: data distribution is normal; H1: data distribution is not normal.

Table 3. Normal Distribution of Research Variables

Variable	Statistics	Significance Level
Follicle Rate	0.493	0.968
Egg Rate at GV Stage	0.963	0.312
Egg Rate at M1 Stage	1.047	0.223
Egg Rate at M2Stage	0.936	0.344
$CYP_{11}A_{1}$Gene Methylation Rate	0.610	0.850

According to the information in Table 3, the significance level of the follicle and egg rates at the GV, M1, and M2 stages and CYP11A1 gene methylation rate were 0.968, 0.312, 0.223, 0.344, and 0.850, respectively. Since these values were higher than0.05, it was concluded that all the variables had normal distribution.

4.2.3. Hypothesis Testing

The research hypotheses were tested using the Pearson's correlation-coefficient, and the obtained results regarding hypotheses 1-4 are shown in Tables 4-7.

Table 4. Pearson's Correlation-coefficient to Test Hypothesis One

Variable	Histone Methylation Ratein ***$CYP_{11}A_1$***Gene
Follicle Rate	-0.181
Significance Level (14)	0.473
Sample Size	18
Result	Rejected

Table 5. Pearson's Correlation-coefficient to Test Hypothesis Two

Variable	Histone Methylation Ratein ***$CYP_{11}A_1$***Gene
Egg Rate at GV Stage	-0.371
Significance Level (14)	0.261
Sample Size	11
Result	Rejected

Table 6. Pearson's Correlation-coefficient to Test Hypothesis Three

Variable	Histone Methylation Ratein ***$CYP_{11}A_1$***Gene
Egg Rate at M1 Stage	-0.152
Significance Level (14)	0.589
Sample Size	15
Result	Rejected

Table 7. Pearson's Correlation-coefficient to Test Hypothesis Four

Variable	Histone Methylation Ratein ***$CYP_{11}A_1$***Gene
Egg Rate at M2 Stage	-0.107
Significance Level (14)	0.672
Sample Size	18
Result	Rejected

Discussion

Histone modifications are one of the epigenetic mechanisms that are used to regulate gene expression without the alteration of gene sequences.Histone acetylation and methylation are reversible states, which could alter the interactions of non-histone proteins with chromatin, thereby changing the chromatin structure and gene expression (13).Histone acetyl transferase and deacetylaseregulate the access of transcription factors to the DNA through lysine acetylation and deacetylationin histone proteins, thereby regulating gene expression. While histone deacetylation is associated with extinction, histone acetylation is related to transcriptional activation.

According to the literature, H3 histone methylation in lysine 9 (H3K3) and lysine 27 (H3K27) is associated with transcriptional suppression, while methylation in H3K4, H3K36, and H3K79 is associated with transcription activation (14). In general, these changes regulate the genome function through the regulation of chromatin availability and compaction without interfering with the DNA nucleotide sequence. Activated chromatin (euchromatin) has low histone methylation and DNA methylation and high histone acetylation with an open structure, which allows access to the transcription factors and polymerase enzymes.Moreover, the tight packaging of chromatin (heterochromatin) contains DNA, hypermethylated histones, and low levels of acetylation and is transcriptionally inactive.

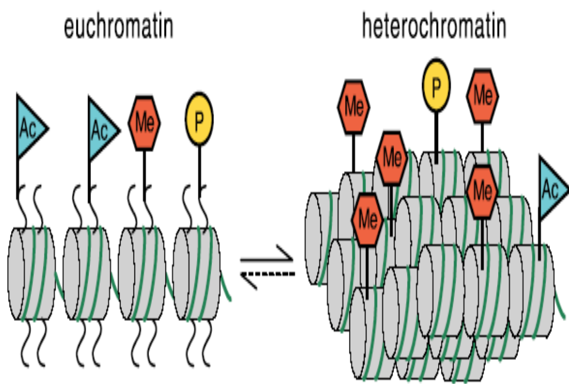

Figure 4. Heterochromatin and Euchromatin Regions

Histone modifications are considered to be the key regulators of gene expression in many diseases, such as gynecological cancers. However, inadequate research has been focused on the role of these modifications in these diseases and infertility (15). In this regard, H3K4me is a widely recognized histone marker, which is involved in gene activation. In addition, H3K4 methylation plays a pivotal role in gene expression (16), and the increase in this histone mark is known as a transcriptional activator.

The CYP11A1 genecodes the P450 cytochrome protein and has an enzymatic role. It is also involved in progesterone synthesis, and the expression of this gene increases rapidly after the LH peak. The rapid change in the expression of these genes facilitates progesterone production by switching the estrogen synthesis to progesterone. These functional changes in steroidization play a key role in the follicle rupture and yellow body formation (17).

According to the literature, histone modification results in the rapid induction of the StAR and CYP11A1 genes and inhibition of CYP19A1 in luteinized granulosa cells during ovulation in mice (18). On the other hand, there has been no research on the epigenetic alterations of the CYP11A1gene in the human granulosa cells, and this is the first research in this regard. However, some studies have been conducted on animal models. For instance, Norihiro et al. assessed the histone modifications in the promoter region of the CYP11A1gene using theChIP technique after the injection of chorionic gonadotropin. The mentioned study was performed on the proximal and distal regions of the CYP11A1 gene promoter.

According to the study by Monterioet al. (2014), H3K27me3 and H3K4me3 histone marks play a key role in the regulation of some genes during fetal development. In the mentioned research, increased H3K4me histone mark was recognized as a transcription activator, while H3K27me is among the histone changes that suppress gene expression; these histone marks create a bivalent domain (15). However, this bivalent domain has two issues; first, H3K27me3 and H3K4me3are rarely involved in the suppression of gene activity. Second, either of these two histone modifications that are more frequent than the other has the dominant impact (17).Therefore, it is essential to examine the effects of these modifications together.

The results obtained from the evaluation of the H3K4me3 histone changes in the CYP11A1 gene regulatory region in the healthy and infertile women in dicatedno significant difference between these subjects regarding the H3K4me3 level. In addition, no significant correlation was observed between H3K4me3 methylation and follicle and egg rates in the subjects. The egg quality at

various stages of meiosis division in oogenesis had no significant association with H3K4me3 histone methylation. In general, the presence of methylated histone in the CYP11A1 gene promoter region had a relatively high rate. Considering the involvementof the CYP11A1 gene in the steroidogenic pathway and progesterone synthesis, and since its expression in granulosa cells (especially luteinized granulosa cells) results in the production of progesterone, it could be inferred that the high rate of H3K4me3 methylation in the regulatory region of the gene is correlated with its expression.

Since the higher number of follicles has a more significant effect on egg maturation and quality and the secretion of steroid hormones increases the growth of follicles, our study was performed on the genes that are involved in steroidogenesis to assess their correlation with the follicle rate and egg maturation. However, the obtained results showed no significant correlation between the epigenetic changes in the CYP11A1 gene promoter and follicle rate. Therefore, it could be concluded that other genes than those considered in the present study are also involved in the steroidization pathway. Furthermore, epigenetic changes might be associated with increased follicle rate and egg quality, which requires further evaluation.

Our findings are in line with the previous studies regarding the presence of histone methylation in the CYP11A1 gene promoter region. However, the former studies in this respect have been conducted on animal models, and data is scarce on the effects of histone methylation on follicular changes and follicle rates.

Recommendations for Further Investigations

1. Evaluation of H3K4me3 histone modifications in CYP11A1 gene promoter in granulosa cells through the injection of gonadotropin hormones to the cells and comparison of the methylation levels before and after hormone injection;

2. Simultaneous evaluation of varioushistone marks and their concomitant effects on CYP11A1 gene expression

Conflict of interest:
The authors declare that there is no conflict of interest.

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