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Histone Methylation in StAR Gene Promoter Using Follicular Granulosa Cells Extracted from the Women Referring to a Fertility Clinic in Tabriz, Iran

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Abstract

Infertility is a disorder of the reproductive system, which often occurs after one year of regular unprotected intercourse with the aim of pregnancy. Several physical functions require the synthesis of steroid hormones, in which gonadal steroids (estrogen and progesterone) play a pivotal role in reproduction. Follicular growth and ovulation depend on the proliferation and differentiation of the granulosa and theca cells, which are possible in the steroid pathway after stimulation with the ovarian gonadotropins and cytokines. Steroidization is initiated with the transfer of cholesterol by the StAR protein to the mitochondrial membrane of the steroid cells, which is followed by a cascade of steroid hormones. Recent studies have highlighted the impact of epigenetic mechanisms on reproduction, emphasizing the importance of these changes in the early and secondary stages of gametogenesis. To determine the causes of infertility, it is essential to recognize the altered epigenetic modifications of the relevant gene and its mechanisms. In the present study, the H3K4me3 methylation level was evaluated in the StAR gene regulatory region in the granulosa cells collected from the fertile and infertile women referring to Tabriz Jihad Infertility Centerin Tabriz, Iran using ChIP-qPCR. According to the results, the H3K4me3 methylation level increased in the StAR gene regulatory region in the fertile women compared to the infertile women. In addition, a significant correlation was observed between the follicle and egg rates at the MII stage and the level of this methylation.

Keywords: Infertility, Epigenetics, Histone Methylation, StAR Gene Regulatory Region

Introduction

Infertility is a complex pathophysiological disorder, which are triggered by several specific physical and physiological agents (1). Infertility is classified as primary and secondary (2). With the advancement in molecular biology, several studies have recently identified the influential mechanisms in reproductive disorders. Apart from genetic and chromosomal abnormalities, defects in sex steroid hormone biosynthesis affect the alteration of sexual maturation and reproductive function (3). The synthesis of all the steroid hormones in the mitochondria of the normal cholesterol precursor is a common feature of various steroidogenic organs (4), where the process of steroidogenic acute regulatory protein (SrAR) is responsible for the transfer of cholesterol to the inner mitochondrial compartment (5). In addition to the main pathways with specific transcription factors, epigenetic mechanisms are involved in the promoter regions of this gene in the regulation of StAR gene expression.

Epigenetics is defined as the molecular factors and processes around the DNA, which regulate genome activity independent of the DNA sequence and are mitotically or meiotically stable (6). Common epigenetic mechanisms include DNA methylation, histone modification, chromatin rearrangement, and non-coding RNAs (7). These dynamic modifications of DNA and histones play a key rolein transcription regulation (8). Due to the different climatic, environmental, and genetic conditions that are involved in the high diversity of gene patterns and promoters in various cells and species (even in similar cells and species), it remains unclear how these changes occur in specific promoter regions and genes. Therefore, many aspects of epigenetics remain unknown. Given the importance of this issue in the regulation of gene expression and the extent of epigenetic processes in the development of specific diseases and cancers, more extensive studies are requiredto address these issues.

Research Background

To date, several studies have shown the effect of the histone modifications of the StAR gene promoter on animal models. For instance, Christenson et al. (2001) investigated the H3 acetylation gradient in the StAR promoter of a monkey species, reporting an increase in the H3 acetylation gradient of the granulosa cells after ovulation induction compared to the non-luteinised granulosa cells. In addition, a rapid reduction was observed in the H3K9 methylation gradient in the promoter cells of the yellow body granulosa (9).

In another research, Shimizu et al. (2009) evaluated the gene expression and transcription factors of steroidogenesis during the luteal stages of the granulosa cells that were isolated from small bovine follicles. The obtained results confirmed the key role of the increased HS acetylation of the StAR gene promoter in the gene expression, as well as the reduced transcription of DAX1, in the synthesis of progesterone in the yellow body of the granulosa cells (10).

In another research, Yamashita H et al. (2011) showed the reverse mode of the test results obtained by Shimizu. This group of bovine granulosa cells was cultured on the BMP-4 and BMP-4-free media. According to the findings of the mentioned research,

progesterone secretion was inhibited due to the inhibition of the H3 acetylation of the StAR promoter in the presence of BMP-4 and suppression of the StAR protein expression. Moreover, the ineffectiveness of BMP-4 in granulosa cell proliferation was reported (11).

Materials and Methods

In the present study, the chromatin immune precipitation (ChIP) technique was applied to evaluate the presence of the epigenetic factor in the StAR gene regulatory region. In this technique, the interaction between a particular protein and specific regions of the genome was examined. Finally, real-time polymerase chain reaction (PCR) was performed for accurate detection.

1.3. ChIP Implementation Stages

Stage 1: Cell fixation and cross-linking;

Stage 2: Cell lysis and chromatin shearing;

Stage 3: Deposition of protein-bound chromatin with the addition ofH3K4me3anti-histone antibody (immune precipitation of cross-linked chromatin);

Stage 4: DNA purification;

Stage 5: Primer design in the region of the target gene promoter and quantitative Qpcr.

Results

4.1. Normalization and Evaluation of the Epigenetic Factor in the StAR Gene Regulatory Region Using the ChIP Technique

In order to perform the Chip examinations, we had to normalize the IP samples containing chromatin, which were bound to the antibodies of H3K4me3-specific epigenetic marker and input samples containing total chromatin. The literature review indicated the lack of consensus on the normalization of the ChIP-qPCR data. Therefore, various normalization methods were used in this regard, and the results were estimated using the input percentage method, SPSS, and statistical tests. Moreover, the qPCR signals that were obtained from the ChIP samples were extracted from the qPCR signals, which had been obtained from the input samples at the outset of the ChIP method, and placed in the following equation:

Input= AE^ (CtPinput – CtPChIP) x Fd x 100%

Where AE is the duplication efficiency, and Fd is the dilution compensation factor to the balance differential input of the DNA quantities and ChIP obtained for the qPCR (value estimated at 0.1 based on the ChIP process). The input percentage of the study samples (presence level of H3K4me3) is depictedin Figures 1-3.

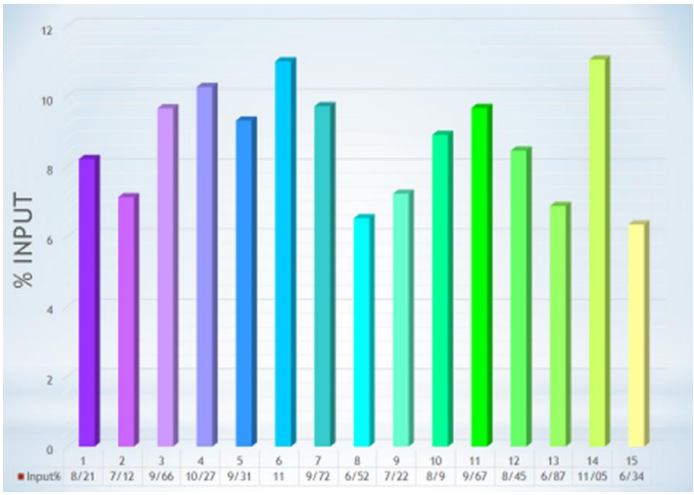

Figure 1. Methylation Rateof Infertile Women

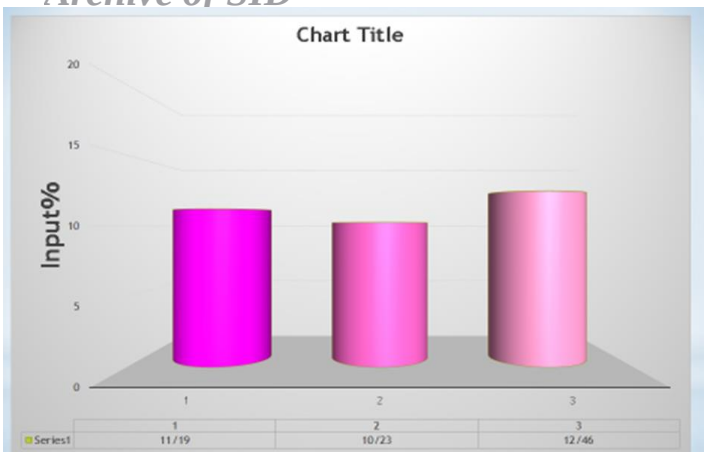

Figure 2. Methylation Rate of Fertile Women

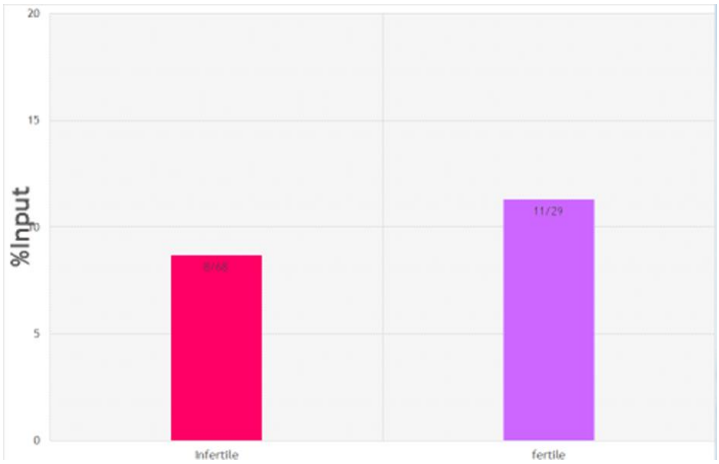

Figure 3. Comparison of Methylation Rate of Infertile and Fertile Women

4.2. Statistical Analysis

The medical files of the fertile and infertile women referring to East Azerbaijan ACECR Fertility Clinic in Tabriz, Iran were used to evaluate the associations between the H3K4me3 methylation gradient of the StAR gene regulatory region and follicular and oocyte rates in the GV, MI, and MI stages using the ChIP method.

4.2.1. Descriptive Statistics of the Quantitative Research Variables

Table 1 shows the descriptive statistics of the quantitative research variables, including the number of the follicles and eggs at the GV, MI, and MII stages and methylation rate of the StAR gene.

According to the information in Table 1, the mean number of the follicles and eggs at the GV, MI, and MII stages and methylation rate of the StAR gene were 24.22±11.97, 4.36±3.44, and 2.60±1.64, respectively.

4.2.2. Normal Distribution of the Research Variables

Table 2 shows the results of the Kolmogorov-Smirnov test regarding the null and alternative hypotheses of the test for the research variables.

Table 1. Descriptive Statistics of Quantitative Variables

Variable	N	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 MI Stage	15	2.60±1.64	1-7
Egg Rate at MII Stage	18	17.17±9.90	4-46
Methylation Rate of StAR Gene	18	9.12±1.80	6.34-12.46

Table 2. Normality of Research Variables

Variable	Statistic	Significance Level
Follicle Rate	0.493	0.968
Egg Rate at GV Stage	0.963	0.312
Egg Rate at MI Stage	1.047	0.223
Egg Rate at MII Stage	0.936	0.344
Methylation Rate of StAR Gene	0.565	0.907

According to the information in Table 2, the follicle rate and egg rate were significant at the GV, MI, and MII stages, and the methylation rateof the StAR gene was estimated at 0.968, 0.312, and 0.223, respectively. Since the mentioned levels were higher than 0.05, all the variables had normal distribution.

4.2.3. Hypothesis Testing

In the present study, the research hypotheses were tested using the correlation-coefficient test. Considering the normal distribution of all the variables, the hypotheses were assessed using Pearson's correlation-coefficient, and the obtained results on hypotheses 1-4 are shown in Tables 3-6, respectively.

Table 3. Results of Pearson's Correlation-coefficient to Test Hypothesis One

Variable	Histone Methylation Rate of StAR Gene
Follicle Rate	0.704
Significance Level	0.001
Sample Size	18
Result	Hypothesis One Confirmed

Table 4. Results of Pearson's Correlation-coefficient to Test Hypothesis Two

Variable	Histone Methylation Rate of StAR Gene
Egg Rate at GV Stage	0.221
Significance Level	0.513
Sample Size	11
Result	Hypothesis Two Ruled Out

Table 5. Results of Pearson's Correlation-coefficient to Test Hypothesis Three

Variable	Histone Methylation Rate of StAR Gene
Egg Rate at MI Stage	0.237
Significance Level	0.396
Sample Size	15
Result	Hypothesis Three Ruled Out

Table 6. Results of Pearson's Correlation-coefficient to Test Hypothesis Four

Variable	Histone Methylation Rate of StAR Gene
Egg Rate at MII Stage	0.788
Significance Level	0.000
Sample Size	18
Result	Hypothesis One Confirmed

Discussion

Histone changes determine the epigenetic structure of chromatin (12). Among various changes, histone methylation is relatively stable in lysine and arginine residues, which are considered as the potential signs of epigenetic information transfer (13).The effects of the methylation of specific amino acids on the structure of histones dependon the type of amino acids and their position in various regions of chromatin (14). According to the literature, H3 and H4 or H3K4me3acetylation activate transcription by opening the chromatin structure, while H3K4me9and H3K4me27 disables transcription through the compression of the chromatin structure (15). Several studies have highlighted the key role of histone methylation in the regulation of chromatin structure and gene expression in follicle maturation, especially meiotic oocytes. Moreover, histone-lysine N-methyltransferase is significantly involved in the rapid progress of meiotic processes through H3K9me1 and H3K9me3 methylation. H3K9me3 emerges in the egg growth process, and growing oocytes are detected in the primary antral follicles, the amount of which increases during the growth stages and is maintained during the maturation and activation of meiotic oocytes. Meanwhile, various types of histone methylation have been documented in granulosa cells and follicle oocytesat various stages of the growth of antral follicles, including H3K4me, H3K4me2, and H3K4me3(16).

Previous studies have shown that histone changes are associated with the rapid induction of StAR and CYP11a1 gene expression and inhibition of CYP19a1 in luteinized granulosa cells during ovulation in mice. To date, no studies have been focusedon the epigenetic changes in human granulosa cells, and the present study was the first research in this regard. However, multiple studies have been conducted on animal models.

In a research by Norihiro Sugino et al. (2012), increased H4 acetylation (H3K4me3) and decreased H3K9me3and H3K27me3 were reported in the StAR promoter after the evaluation of the DNA methylation structure and promoter histone modifications of the StAR and CYP19a1genes after the injection of human chorionic gonadotropin (hCG) (17-19). Finally, the rapid induction of StAR gene expression was reported, along with the decreased chromatin density of the luteinizing granulosa cells after LH increase; notably, the reverse mode of the mentioned issues has been recorded for the CYP19a1 gene(17). In 2016, the same researchers evaluated and compared histone changes in two genes of CYP11a1 (gene encoding the p450 cholesterol chain, which is involved in the first stage of progesterone synthesis) and StAR, demonstrating that the acetylation level of the CYP11a1 gene promoter was similar to that of the StAR gene promoter, while in the reverse form of the CYP19a1 gene promoter after hCG injection (18).

In another study performed in 2016, the mentioned researchers reported significant changes in the expression of various enzyme genes, which are involved in the histone modifications of the luteinizing granulosa cells in rats after the stimulation of ovulation with chorionic gonadotropin, and the expression of various genes was affected in

the early luteal stages and during the formation of the yellow body (19).

In another study, Schwarzenbach H. et al. (2003) evaluated the articles published on increased StAR gene expression and progesterone production due to the stimulation of chorionic gonadotropin, reporting the direct effects of progesterone on the StAR gene expression. Furthermore, the researchers introduced the steroid load as the stimulating factor in the expression of this gene (20). In the present study, H3K4me3methylation level increased in the fertile women compared to the infertile women. In addition, a significant correlation was observed between the presence of H3K4me3 and increased follicle and egg rates at the MII stage. Nonetheless, no changes were observed in the egg rates at the GV and MI stages. In this regard, our findings are in line with the results of the previous studies.

Considering that one of the main goals of steroid hormones is to promote the process of folliculogenesisand histone changes occur at each stage of gametogenesis and development, and since this type of histone methylation is observed in the granulosa cells, follicle oocytes, and various stages of the growth of primary to the antral follicles, it could be inferred that the presence of such methylation increases the growth of follicles and oocytes. Therefore, increased follicle and egg rates at the MII stage were affected by the H3K4me3 presence in the current research.

Considering the complexity of molecular mechanisms and cascaded signals of the steroidization pathway, as well as the enzymes and factors involved in the pathway that act dependently (defects in the genes encoding of each of these enzymes disrupt the steroidization process), and since all these genes are affected by the epigenetic mechanisms in their position, further investigations are required to confirm this issue. Furthermore, the correlation between epigenetics and human reproduction may represent an interesting field of research (21).

Conclusion

Currently, infertility diagnostic methods and available treatments do not respond to all infertility cases. Therefore, epigenetic aspects should also be taken into account in the examination of infertile individuals. Since the correction of these factors is easier than genetic correction owing to the dynamic nature of the epigenetic pattern, proper management of epigenetic events and thorough understanding of the impact of these mechanisms could contribute to the effective treatment of infertility, as well as the prevention of this issue. On the other hand, the role of epigenetic factors in infertility and effects of assisted reproduction on the epigenetic pattern of the fetus have not been addressed in studies for many years. Recently, epigenetic factors have become an important and measurable factor in infertility. Therefore, the further study of these factors in the near future enables the development of epigenetic drugs in order to correct epigenetic processes in infertile individuals. As such, these factors must be explored broadly and incorporated into the diagnostic methods used for infertility.

Declarations

Conflict of interest

We declare that we have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the article.

Authors' contributions

All authors contributed equally to this work.

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