DNA METHYLATION USING IMPRINTING SNRPN GENE AND EFFECT ON ARRESTED SPERMATOGENESIS IN IRAQI OLIGOSPERMIA PATIENTS

Halah Kamal Al-Qazzaz¹ and Salwa Jaber Al-Awadi²

College of Biotechnology, Al-Nahrain University, Baghdad, Iraq.

Abstract

DNA methylation process is a very essential step during sperm maturation through facilitating the replacement of histone by transition nuclear proteins, and then, replaced by protamine during the spermatid elongation stage. The present study was designed to investigate the association between the methylation patterns of SNRPN imprinting control region (ICR) and male infertility (MI). One hundred of semen and blood samples were collected from [50 infertile patients (oligospermia) (OZ) and 50 fertile patients (normal control) (NZ)]. Semen samples (100) were collected for microscopic examination of seminal fluid analysis (SFA) for diagnosis male infertility and for molecular study using specific primer of Imprinting SNRPN gene DNA methylation by Quantitative Real-time Polymerase Chain Reaction (qPCR). Blood samples (100) were collected for hormonal level assay (Follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and testosterone) by ELISA Kits. This study revealed that the SNRPN hypermethylation pattern was a higher significant difference for infertile patients compared with fertile patients. The prepares of this study to evaluate methylation patterns levels in an infertile patient using qPCR technique and effect on sperm parameters.

Key words: DNA methylation, Imprinting gene, Male infertility, SNRPN, Spermatogenesis.

Introduction

DNA methylation is a dynamic mechanism of gene expression regulation that occurs on dinucleotides formed by cytosine and guanine are grouped in so-called differently methylated regions (DMRs), that are often placed near gene regulatory regions, such as the promoter (Chen et al., 2016; Hao et al., 2016). It is defined as the addition of a methyl group (CH3) to the fifth position of the cytosine in CpG dinucleotides, where the S-adenosylmethionine is used as a donor for the methyl group (Schübeler, 2015). Indeed, specific CpG sites in a single cell can be found in a methylated, or an unmethylated state (Zaghlool et al., 2015). DNA methylation is one of the most commonly studied epigenetic regulation mechanisms and is involved in the regulation of many biological processes. One of the main roles of epigenetic modifications through DNA methylation is to control gene transcription in response to external and internal stimuli by targeting specific regulatory DNA motifs, such as promoter and enhancer regions (Nasri et al., 2017). The methylation processes are regulated by DNA methyltransferases enzymes (DNMTs) (Xia et al., 2015). The gene suppression is correlated with hypermethylation of CpG islands, while the activity of gene expression is correlated with a decline in the methylation level of DNA in the CpG islands (Heyn et al., 2016). Hypermethylation suppresses gene expression since methyl groups prevent the recruitment of transcription factors and DNA polymerases (Lu et al., 2015). Conversely, hypomethylation promotes gene expression (Uysal, et al., 2016; Kenny, 2018). DNA methylation occurs in primordial germ cells (PGC) and subsequently takes place during the maturation of germ cells and spermatogenesis before germ cells enter meiosis (Akane et al., 2015). During the maturation of germ cells and spermatogenesis, errors in the process of methylation erasure, reestablishment, and maintenance can lead to disorders.

¹Author for correspondence: E-mail: hallakamal83@yahoo.com
in genetic imprinting and thereby may result in aberrant spermatogenesis and impaired sperm function (Dong et al., 2016; Filippo et al., 2019). SNRPN (small nuclear ribonucleoprotein polypeptide N) hypermethylation of the maternally imprinted gene was associated with decreased sperm motility and percentage of morphologically normal sperm (Camprubi et al., 2016; Du et al., 2016). Multiple sperm defects usually occur together in the clinic, and there are fewer patients with a single-factor phenotype. Whether certain abnormal methylation patterns of one or multiple specific CpG sites within the imprinted gene SNRPN is pathological or potential diagnostic biomarkers for multiple sperm defects (Hongli et al., 2018). The current study was designed to evaluate the variation in the SNRPN DNA methylation level in oligospermia and normospermia and their effect on semen parameters.

**Materials and Methods**

**Study design**

The study design case-control study one hundred of male participants (50 samples from infertile males as Oligospermia patients (OZ) according to the World Health Organization guidelines (WHO, 2010) and 50 samples from fertile males as a control group (NZ)) with an age range from (20-50) years. Subjects samples were collected from Kamal Al-Samurai Specialized Hospital for Infertility and IVF, 2019. Semen samples (100) were collected by masturbation after 3-7 days of abstinence for diagnosis after 30 min liquefaction, then analyzing for microscopic examination of seminal fluid analysis (SFA) and for molecular study DNA methylation pattern as described by Al-Haboubi (2018). Blood samples (100) were collected by masturbation after 3-7 days of abstinence for diagnosis after 30 min liquefaction, then analyzing for microscopic examination of seminal fluid analysis (SFA) and for molecular study DNA methylation pattern as described by Al-Haboubi (2018). Blood samples (100) were collected by masturbation after 3-7 days of abstinence for diagnosis after 30 min liquefaction, then analyzing for microscopic examination of seminal fluid analysis (SFA) and for molecular study DNA methylation pattern as described by Al-Haboubi (2018).

**DNA Isolation**

Total DNA was isolated from semen fluid samples using the genomic DNA purification protocol kit (RIBO-Sorb-AM) (AmpliSens, Russia) according to the manufacturer’s instructions. The quantity and quality of the isolated DNA were estimated the purity by Nanodrop (Essaada et al., 2015).

**DNA Methylation Assay**

DNA was extracted from the semen samples by using specific extraction kit. The genomic DNA (gDNA) were converted to methylated DNA patterns using Bisulfite conversion methylated DNA EpiTect (Qiagen, Germany) according to the manufacturer’s instructions (He et al., 2013). Bisulfite conversion was used for methylation-specific PCR (q-MSP) analysis. The q-MSP program consisted of the following condition, denaturation step 95 ºC for 5 min and incubation 60 ºC for 25 min in hold1 followed by denaturation step 95 ºC for 5 min and incubation 60 ºC for 85 min in hold 2, then denaturation step 95 ºC for 5 min and incubation 60 ºC for 175 min in hold 3, followed by, 20 ºC indefinite in hold 4. The next step DNA purified Bisulfite followed the manufacture protocol treatment. Primers for the SNRPN gene promoter using the international databases (NCBI). The SNRPN forward primer F:5¹- TTAGGTT GTTTTTTAGAGAAG-3¹ and SNRPN reverse primer R:5¹- CCTACACTACRACAAACAAAC-3 ¹.

**Quantitative Real-time Polymerase Chain Reaction Analysis (Q-PCR)**

The q-PCR program of SNRPN gene as the following condition: The denaturation step 94 ºC for 3 min for 1 cycle in hold 1, followed denaturation step 94 ºC for 30 sec, then annealing 60 ºC for 30 sec and extension 72 ºC for 30 sec for 35 cycles in hold 2, followed final extension 72 ºC for 5 min for 1 cycle. Rising by 2ºC each step, acquire to High-Resolution Melting [HRM] for SNRPN gene.

**Statistical analysis**

All data were analyzed using SPSS software V26 (SPSS Inc., USA) (2018), € Chi-square test of association was used. The Spearman ‘s test was applied to assess the correlation coefficient analysis. The probability of p<0.05 was considered significantly different.

**Results and Discussion**

The result of age the mean ± SD were 32.44± 7.45 and 31.79 ± 6.79 (P-value=0.650) for OZ and NZ, respectively. This result showed there was no significant difference between OZ and NZ groups in age factor, as well as, the result of duration of a married factor the mean± SD was 4.47 ± 3.58 and 5.26 ± 5.18 for OZ and NZ groups, respectively. This result showed there was
no significant difference between OZ and NZ groups for the duration of the married factor. A similar observation result was reported by Elbashir et al., (2018) and Farah (2019), they found there was no significant difference between infertile and fertile groups on the effect of age and duration of married on infertility status. Conti and Eisenberg, (2016) revealed that in infertility cases the early men’s age, aged was the effect on sperm quality and decrease infertility with increased male age older age and it related with male infertility. Other researchers were found out that when men grow older the genetic defects in sperm do increase and altered, which leads to decreased infertility (Al-Quzwini, 2016; Durairajanayagam, 2019).

The result in table 1 as it clears the microscopical analysis of seminal fluid profile for OZ patients, as shown that the mean ± SD of sperm count there was 9.37 ± 1.3 and 42.10 ± 10.1 for OZ and NZ patients, respectively (P-value= 0.001). On one hand, the mean ± SD of sperm motility 28.50 ± 13.2 and 59.7 ± 10.50 for OZ and NZ patients, respectively (P-value= 0.001). On another hand, the mean ± SD of sperm immotile 77.80±10.1 and 31.4±10.60 for OZ and NZ patients, respectively. The mean ± SD of sperm normal morphology 24.90±11.90 and 67.36±9.91 for OZ and NZ patients, respectively (P-value= 0.001). In contest. the mean ± SD of sperm abnormal morphology 79.60±12.00 and 36.10±11.1 for OZ and NZ patients, respectively (P-value= 0.001). The result of the current study produces that the clinical parameters of the subject’s seminal fluid profile parameters there were highly significant differences between OZ and NZ groups (P-value=0.001). A similar result obtained by Hao et al., (2016) was reported that sperm concentration in the infertile group was significantly lower than in the fertile groups as well as Amir (2018) that found that the lower limit men with semen parameters had a chance of being infertile. Also, Bracke et al., (2018) reported that the incidence of male infertility has increased progressively, semen quality, spermatogenesis, and sperm quality.

On the other hand, the result of the type of infertility for oligospermia patients well be characterized into two types of infertility, primary infertility types the mean± SD was 38 (76%) patients, and secondary infertility type the mean± SD was 12 (24%) patients. As a result, it’s clear, there was no significant difference when camper between primary and secondary infertility types. In spite of this, the primary infertility was higher than secondary infertility in the OZ group. The primary male infertility can result from a variety of conditions including congenital disorders, hypothalamic-pituitary disorders, systemic disorders, chronic illnesses, and nutritional deficiencies thus, it will become more common than secondary infertility (Barratt et al., 2017).

The result of the hormonal assay profile in this study as listed in table 2: as it clears the (mean ± SD) of FSH hormone level (mIU/ml) was 9.29 ± 3.45 and 4.49 ± 1.42 for OZ and NZ patients, respectively. On one side hand, the (mean ± SD) of the LH hormone level (mIU/ml) was 8.21 ± 1.84 and 6.60 ± 0.75 for OZ and NZ patients, respectively. On the other hand, the (mean ± SD) of testosterone hormone level (ng/ml) was 11.17 ± 1.73 and 5.95 ± 1.99 for OZ and NZ patients, respectively. Overall, our findings there were highly significant differences between OZ and NZ patients in the FSH, LH, and testosterone hormone levels. A majority of infertile men within the normal range for FSH, LH, and testosterone levels (Johannes et al., 2019). A similar result has been reported by Afrim et al., (2018), Andreassen, et al. (2018) and Farah, (2019) were found there was increase significant differences in LH and FSH hormone levels. The high level of LH and FSH hormone levels from the feedback mechanism of the hypothalamic-pituitary-gonadal leading to a decrease the sperm count in oligospermia patients (Mahat et al.,2016). On the other hand, in this study increase in the testosterone hormone level of the OZ group when compared with the NZ group. Testosterone evaluation hormone levels were useful in

| Table 1: The description of semen microscopical analysis in study groups. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Sperm Parameters               | OZ group (mean± SD) (n=50) | NZ group (mean± SD) (n=50) | P-value |
| Count (×10⁶/ml)                | 9.37±1.3         | 42.10±10.1      | 0.001***        |
| Motility (%)                   | 28.50±13.2       | 59.7±10.50      | 0.001***        |
| Immotile (%)                   | 77.80±10.1       | 31.4±10.60      | 0.001***        |
| Normal Morphology (%)          | 24.90±11.90      | 67.36±9.91      | 0.001***        |
| Abnormal Morphology (%)        | 79.60±12.00      | 36.10±11.1      | 0.001***        |

Independent t test was used to test between groups N.S: Non-significant, *, **, *** significant at 0.05, 0.01 ,0.001 prob. Level.

| Table 2: Hormonal profile for the studied groups. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Hormones                        | OZ group (mean± SD) (n=50) | NZ group (mean± SD) (n=50) | P-value |
| FSH (mIU/ml)                    | 9.29±3.45       | 4.49±1.42       | 0.001***        |
| LH (mIU/ml)                     | 8.21±1.84       | 6.60±0.75       | 0.001***        |
| Testosterone (ng/ml)            | 11.17±1.73      | 5.95±1.99       | 0.001***        |

Independent t test was used to test between groups N.S: Non-significant, *, **, *** significant at 0.05, 0.01 ,0.001 prob. Level.
Table 3: Correlation Coefficient between methylation level and seminal fluid parameters for OZ.

<table>
<thead>
<tr>
<th>Methylation Level</th>
<th>Sperm Count</th>
<th>Sperm Motility (%)</th>
<th>Sperm Immotile (%)</th>
<th>Sperm Normal Morphology</th>
<th>Sperm Abnormal Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNRPN</td>
<td>r</td>
<td>-0.46**</td>
<td>-0.31*</td>
<td>0.31*</td>
<td>-0.28</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.001</td>
<td>0.05</td>
<td>0.05</td>
<td>0.861</td>
</tr>
</tbody>
</table>

Spearman’s test, r: Correlation coefficient, p-value>0.05: Not significant, p-valued≤0.05: Significant.

Fig. 1: DNA methylation at DMRs of imprinting genes as predicted by HRM software Identification methylated and unmethylated pattern of SNRPN gene promote by HMR using qPCR technique. a: Normalized Graph.

the management of the major regulators of germ cell development and have the main role in male spermatogenesis as reported by Al-Nahi, (2015) and Patel et al., (2018).

The result of the present study for the SNRPN methylation level mean±SD was 52.70± 18.1 and 48.70 ± 13.50 for OZ and NZ groups, respectively. These results as it clarifies there was no significant difference between DNA methylation pattern for OZ and NZ groups (P-value= 0.212). In other words present hypermethylation in SNRPN methylation level in OZ than NZ patients. A similar, result revealed by Poplinski et al., (2010) and Santi et al., (2017). The hypermethylation of SNRPN was associations with low sperm quality has been reported by Botezatu et al., (2014). DNA methylation occurs in primordial germ cells and subsequent in males has been reported by Hao et al., (2016).

The qMSP results in the current study in Fig. (1) as it clears the SNRPN methylation pattern for OZ and NZ groups were produced 62 samples as positive methylation 35 (70%) and 27 (54%) for OZ and NZ groups, respectively and the negative methylation 38 samples 15 (30%) and 23 (46%) samples for OZ and NZ groups, respectively. The result was no significant difference (P-value=0.099). The SNRPN-ICR is a methylated maternal allele. Kobayashi et al., (2017) have been found the imprint methylation errors in sperm were associated with living environmental factors. Then, aberrant methylation and a particular lifestyle were associated with severe Oligospermia. The result of conversion was converted whole-genomic DNA (gDNA) extraction from a seminal fluid sample from OIP and NFP groups to Methylated pattern DNA levels of maternally methylated imprinted SNRPN genes by qMSP shown the methylated and unmethylated patterns in normalized and deference graphs promote by High Resulting Melting Analysis (HMR) using qPCR technique (Houshdaran et al., 2007).

The result of the correlation coefficient between SNRPN methylation level and semen parameters for OZ as it clears in table 3: there was a great negative correlation coefficient between sperm count, sperm motility as well as with sperm normal morphology and SNRPN methylation level. While, in contrast, the present positive correlation coefficient between sperm immotile and SNRPN methylation level. Similar results obtained by El-Hajj et al., (2011) that has been found a significant association of abnormal semen parameters with methylation marks showing also differed significantly between sperm samples from infertile and presumably fertile males. A significant association between male factor infertility hypermethylation of SNRPN in sperm has been reported (Poplinski et al., 2010; Botezatu et al., 2014).

Conclusion

The SNRPN hypermethylation pattern for OZ patients and serve as epigenomic biomarkers for assessment of oligospermia infertility in men with multiple sperm defects.
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