Original Article

Study of Correlation between Serum Vitamin B12 Level and Aberrant DNA Methylation in Infertile Males

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Abstract

Introduction: Altered DNA methylation pattern in sperms has been associated with infertility in males demonstrating defective spermatogenesis or low semen quality. Vitamin B-12, by affecting 1-carbon metabolism pathways, might alter the DNA methylation pattern. We aimed to study the correlation of serum vitamin B12 levels with aberrant DNA methylation in infertile male patients. **Methods:** A cross-sectional study was conducted on 17 oligozoospermic infertile males (WHO criteria, 2010) and 10 healthy fertile males. Serum vitamin B12 levels were estimated using the chemiluminescence method. Global methylation was determined using the ELISA system (Imprint Methylated DNA Quantification Kit, Sigma–Aldrich). The levels of global DNA methylation were calculated and compared relative to the methylated (100%) control DNA provided with the kit. **Results:** Mean serum vitamin B12 concentration in the control group was higher than that of the case group. This difference in serum vitamin B12 concentration in both groups was found statistically significant. Although the results of this study show that oligozoospermic men have relatively lower global DNA methylation as compared to normozoospermic control, the values could not reach a statistically significant level. A small positive correlation was found between serum vitamin B12 levels and percent methylation defect ($r = 0.14$) but was statistically insignificant. **Conclusion:** Our study concludes that oligozoospermic infertile males have a significant deficiency of vitamin B12 as compared to normozoospermic fertile males. This study did not find any significant difference in global DNA methylation between the two groups. The present study does not suggest any correlation between serum vitamin B12 level and percent DNA methylation.

Keywords: Aberrant DNA methylation, infertile male, serum vitamin B12

Introduction

The prevalence of infertility in couples of reproductive age group is 8%–12%.[1] Results from a Global Burden of Disease Study, 2017, showed that the age-standardised prevalence of infertility increased annually by 0.291% in men and by 0.370% in women between 1990 and 2017.[2] Both social and environmental factors are responsible for the increase in the number of patients with infertility. Infertility in the male partner contributes to approximately half of all cases.[3] Male factors solely account for 20%–30% of cases of infertility and are contributory in a further 20%.[4] Male infertility is a complex multifactorial pathological condition and is poorly understood in most cases. Genetic factors account for at least 15% of male infertility.[5] Kallmann syndrome, Klinefelter syndrome, congenital bilateral absence of the vas deferens associated with cystic fibrosis gene mutation, and Y chromosome micro-deletions are examples of primary known genetic causes of male infertility.[6] The most common acquired cause of male infertility is varicocele, with a prevalence of 40%.[7] Male infertility is idiopathic in 30%–50% of cases, with no contributory female infertility.[8] Recently, epigenetic changes in DNA have emerged as the most promising research area in the understanding of male infertility. A large number of studies have shown that epigenetic alterations can also change the quantity and quality of sperm and are responsible for male infertility.[9] One of the best-understood and earliest epigenetic modifications is DNA methylation. DNA methylation plays a critically important role during spermatogenesis. The

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methylation pattern in mature sperm reflects changes in the gene expression pattern that takes place during spermatogenesis. DNA methylation regulates the transcriptional activity of genes, and a normal DNA methylation pattern in germ cells contributes to the progression of meiosis that culminates in the production of mature and functional spermatozoa.[10] Therefore, defects in DNA methylation may affect spermatogenesis and might explain some cases of idiopathic male infertility. Vitamin B-12 is an essential micronutrient of the 1-carbon metabolism pathways. These pathways are required for the donation of methyl groups to the nucleic acids and proteins.[11] In this *S*-adenosylmethionine (SAM) cycle, the most important step is the conversion of *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH), which results in the methylation of important macromolecules in the human body, such as nucleic acids. Many studies have investigated the effect of vitamin B12 on semen quality, and some studies have found that infertile men have lower vitamin B12 concentrations than fertile men.[12] Alterations in the DNA methylation pattern may be a possible mechanism by which vitamin B-12 deficiency affects semen quality. However, the influence of vitamin B12 deficiency on gene-specific DNA methylation is less clearly understood.[13] Therefore, the current project aimed to study the correlation of serum vitamin B12 levels with aberrant DNA methylation in infertile male patients. For the first time, this study sought to investigate the influence of vitamin B12 deficiency on sperm DNA methylation at the genome-wide level.

Materials and Methods

Study design

Cross-sectional study.

Study duration

September 2020–January 2022.

Sampling

Simple random sampling.

Sample size

Cases (n = 17) and control (n = 10).

In the context of this study, which aimed to examine differences between oligozoospermic infertile men (cases) and healthy normozoospermic fertile male controls, the initial plan was to include 20 samples in each group. However, due to the unprecedented challenges posed by the COVID-19 pandemic and the resulting limitations in recruiting physical participants, the final sample size comprised 17 cases and 10 controls.

While a larger sample size is generally preferred for enhanced statistical power and increased precision in estimating effect sizes, the decision to proceed with a smaller sample size in this study was driven by practical considerations and the exploratory nature of the research. In exploratory or pilot studies, where the primary objective is to gather preliminary data and generate hypotheses, smaller sample sizes are often acceptable, particularly when the study population is well-defined and homogeneous. There are several studies conducted with similar or smaller sample sizes on the same population in India and abroad, as mentioned in references.

Furthermore, emphasis was placed on reporting and interpreting effect sizes and confidence intervals rather than solely relying on *P* values. This approach provides more meaningful information about the magnitude and precision of the observed effects, even in the presence of a smaller sample size.

Subjects

Clinical assessment of fertile and infertile individuals was conducted at our institute. The study subjects participated in the study after signing informed consent.

The assessment included personal and family medical history to rule out genetic conditions and physical examinations with special emphasis on sexual characteristics, testes, and genitalia. A detailed clinical history was taken from the subjects regarding regular unprotected intercourse with their partner, symptoms of hypogonadism, smoking history, recent intake of vitamin B12, and partner's menstrual history. A minimum of two semen analyses were performed in accordance with World Health Organization (WHO) criteria 2010.

Inclusion criteria

Infertile men (with oligozoospermia) between 21 and 45 years of age with partners less than 40 years of age with a history of regular sexual intercourse without contraception for ≥1 year without achieving a pregnancy with no history of B12 supplementation in recent 3 months were included in this study.

Exclusion criteria

Men not having regular and unprotected intercourse, with an infertile female partner, with a history of cryptorchidism, hypogonadotropic hypogonadism, Klinefelter's syndrome, vasectomy, cancer, or mumps orchitis were excluded from the study. Men with obstructive azoospermia and with a history of recent vitamin B12 supplementation were also excluded from the study. Patients with overt hypothyroidism or hyperthyroidism were also excluded. Fertile men aged 21–45 years were included as controls in this study.

Sample collection

First, 5 mL of venous blood was collected in an EDTA vial and in a plain vial. Next, serum was isolated from plain vial by centrifugation at 3000 rpm for 10 min at 4°C. Isolated serum samples were stored at −80°C till further analysis.

Semen sample was collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence. Semen analysis was performed using the standard WHO manual for the examination and processing of human sperm. Seminogram was prepared for each individual. Semen data included volume, pH, sperm concentration, motility assessment, vitality, and morphology.

DNA isolation protocol

Genomic DNA was isolated from the ejaculated sperm of both fertile and infertile individuals using the Masture Pure Complete DNA and RNA Purification Kit. First, 1 µL of Proteinase K was diluted into 150 µL of 2X T and C Lysis solution for each sample. Next, 150 µL of 2X T and C Lysis solution containing the Proteinase K was added to the 300-µL semen sample and mixed thoroughly. The solution was incubated at 65°C for 20 minutes and vortexed every 8 minutes. The samples were kept on ice for 5–10 minutes and then proceeded with total nucleic acid (DNA/RNA) precipitation.

Next, 150 µL of MPC protein precipitation reagent was added to 350 µL of lysed sample and vortexed vigorously for 10 seconds. The debris was pelleted by centrifugation at 4°C for 15 minutes at ≥12,000 × g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube and the pellet was discarded.

Then, 500 µL of isopropanol was added to the recovered supernatant. The tube was inverted 30–40 times. DNA was pelleted by centrifugation at 4°C for 15 minutes in a microcentrifuge. The isopropanol was carefully poured off without dislodging the DNA pellet. The pellet was rinsed twice with 70% ethanol. All the residual ethanol was removed using a pipette. Total nucleic acids (DNA) were re-suspended in 35 µL of TE buffer. The amount of DNA/RNA in the samples was quantified with the help of Nanodrop 2000. Next, 1 µL of the sample was kept on the stage of the Nanodrop 2000 instrument. The purity was assessed using (A260/280 ≥ 1.8; A260/230~2.0-2.2).

Estimation of Vitamin B12 levels

Serum vitamin B12 levels were estimated using the chemiluminescence method (Beckman Coulter UniCel DxI 600, California, USA).

Estimation of DNA methylation

Global methylation was determined using the ELISA system (Imprint Methylated DNA Quantification Kit, Sigma–Aldrich). The levels of global DNA methylation were calculated and compared relative to the methylated (100%) control DNA provided with the kit.

Statistical analysis

Data were recorded on a Microsoft Excel spreadsheet, and data cleaning was done subsequently. All statistical analyses were performed using MINITAB 16 (Minitab Inc., State College, Pennsylvania, USA). Distributions of data sets obtained in the study were checked for normality by using the Kolmogorov-Smirnoff test and homogeneity of variances by using Bartlett's test for equal variances. Comparative analysis between the control group and the study group was performed using unpaired *t*-test followed by Tukey's post-hoc comparison of means. Pearson's correlation coefficient was calculated to assess the correlation between serum vitamin B12 and percent DNA methylation. A *P* value of ≤ 0.05 was considered statistically significant.

Ethical aspects

The study was approved by the institutional ethics committee vide letter no Dean/2020/EC/2151 dated 24.09.2020. Written informed consent was obtained for participation in the study and use of the patient data for research and educational purposes guidelines laid down in declaration of Helsinki (1964) and as revised later were followed.

Results

The study included 17 oligozoospermic infertile men fulfilling the inclusion criteria and 10 healthy normozoospermic fertile male controls.

The mean age of participants in the case group was 30.2 ± 1.2 years and in the control group was 30.4 ± 1.1 years with no significant difference between them (t = 0.14; *P* = 0.891; df = 14) [Table 1]. The oldest participant in the case group was 42 years old (range: 22–42 years) and in the control group was 38 years old (range: 24–38 years). In this study, the mean sperm count (million/mL) in the control group was 54.70 ± 4.13, while in the case group, it was 7.71 ± 0.87. This difference in sperm count in both groups was highly significant (t = 11.15; *P* < 0.0001; df = 9) [Table 1, Figure 1]. Out of 17 infertile males, six males (29.41%) had mild oligozoospermia (sperm count: 10–15 million/mL), six males (35.29%) had moderate oligozoospermia (6–10 million/mL), and five males (29.41%) had severe oligozoospermia (<5 million/mL). In the case group, the mean haemoglobin concentration was 13.13 ± 0.25 g/dL, while in the control group, it was 14.89 ± 0.34 g/dL. Between the two groups, haemoglobin concentration differed significantly (t = 4.17; *P* = 0.001; df = 18) [Table 1, Figure 2]. However, the statistical results did not show any significant variation in the mean corpuscular volume (MCV) (t = 0.56; *P* = 0.579; df = 22) of the two groups. In this study, the mean value of haemoglobin in the control group was higher than that in the case group. The

Table 1: Comparison of clinical and biochemical parameters between cases and controls among study subjects

Parameters	Control group (*n*=10)	Case group (*n*=17)	*t*-test (unpaired)
Mean age (years)	30.4±1.1[a]	30.2±1.2[a]	*P*=0.891
Sperm count (Million/ ml)	54.70±4.13[b]	7.71±0.87[a]	*P*<0.0001
LH (mlU/mL)	4.76±0.34[a]	5.59±0.35[a]	*P*=0.105
FSH (mlU/mL)	5.16±0.48[a]	6.80±0.80[a]	*P*=0.091
Testosterone (ng/dL)	471.30±23.41[a]	481.76±44.38[a]	*P*=0.837
Vitamin B12(pg/mL)	354.70±30.40[b]	101.94±17.98[a]	*P*<0.0001
Haemoglobin (g/dL)	14.89±0.34[b]	13.13±0.25[a]	*P*<0.001
Mean corpuscular volume (fl)	96.15±2.24[a]	94.41±2.13[a]	*P*=0.579
Percent DNA methylation	26.04±5.03[a]	20.33±3.78[a]	*P*=0.376

[a]Statistically insignificant (*P*>0.001). [b]Statistically significant (*P*<0.001)

mean concentration of luteinizing hormone (LH, mlU/mL) in the case group was 5.59 ± 0.35, while in the control group, it was 4.76 ± 0.34. Although the mean concentration of LH was higher in infertile males, this difference was not statistically significant (t = 1.69; *P* = 0.105; df = 23). The mean concentration of follicle-stimulating hormone (FSH, mlU/mL) in the case group was 6.80 ± 0.80, while in the control group, it was 5.16 ± 0.48. The concentration of FSH was higher in infertile males as compared to fertile males, but this difference was not statistically significant (t = 1.76; *P* = 0.091; df = 24). However, this difference was toward statistical significance (*P* = 0.091). The mean concentration of testosterone (ng/dL) in the case group was 481.76 ± 44.38, whereas in the control group, it was 471.30 ± 23.41; this difference was not statistically significant (t = 0.21; *P* = 0.837; df = 22) [Table 1]. In this study, the mean serum vitamin B12 concentration in the control group was higher than that in the case group. In the case group, the mean serum vitamin B12 concentration (in pg/mL) was 101.94 ± 17.98, while in the control group, it was 354.70 ± 30.40. This difference in serum vitamin B12 concentration in both groups was highly significant (t = 7.16; *P* < 0.0001; df = 15) [Table 1, Figure 3]. In this study, the mean % DNA methylation in the control group was 26.04 ± 5.03, while in the case group, it was 20.33 ± 3.78. Although the results of our study show that oligozoospermic men have relatively lower global DNA methylation as compared to normozoospermic control, the values could not reach a statistically significant level (t = 0.91; *P* = 0.376; df = 18) [Table 1, Figure 4]. Pearson's correlation coefficient was calculated to assess the correlation between serum vitamin B12 levels and percent methylation defect. A small positive correlation was found between serum vitamin B12 levels and percent methylation defect (r = 0.14). However, this correlation was not found significant (*P* = 0.76) [Figure 5].

In the present study, between the two groups, namely the control group and the case group, the results of the unpaired *t*-test revealed that the sperm count (t = 11.15; *P* < 0.0001; df = 9), the concentration of serum vitamin B12 (t = 7.16; *P* < 0.0001; df = 15), and the concentration of blood haemoglobin (t = 4.17; *P* = 0.001; df = 18) differed significantly. Tukey's post-hoc comparison of means further exposed that between the two groups, the sperm count (54.7 ± 4.12 million/mL), the concentration of serum vitamin B12 (354.70 ± 30.40 pg/mL),

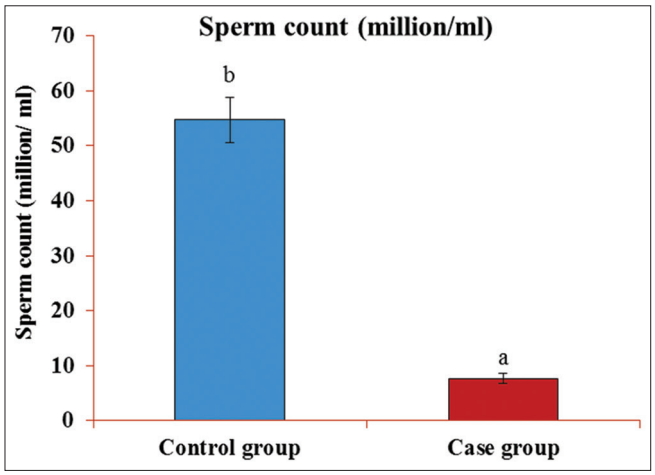

Figure 1: Sperm count in the subjects included in the study (values are Mean ± SE; small letters (a and b) represent a comparison of means based on Tukey's post-hoc test)

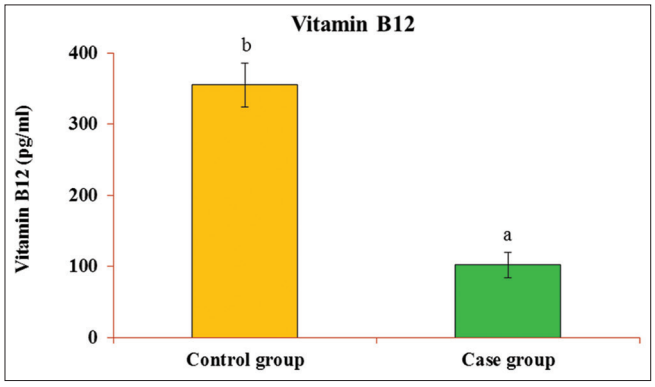

Figure 3: Serum Vitamin B12 concentration in the subjects included in the study (values are Mean ± SE; small letters (a and b) represent a comparison of means based on Tukey's post-hoc test)

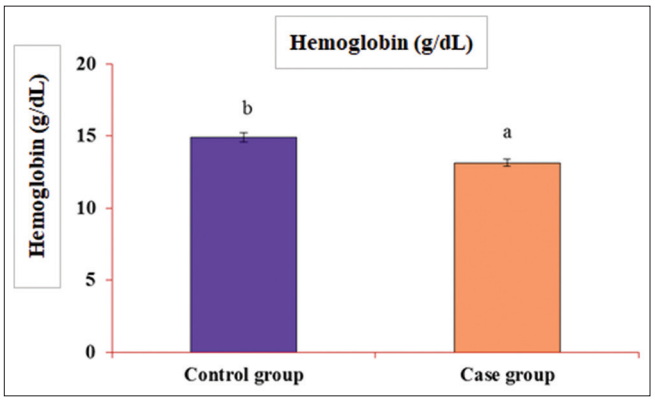

Figure 2: Haemoglobin concentration in subjects included in the study (values are Mean ± SE; small letters (a and b) represent a comparison of means based on Tukey's post-hoc test)

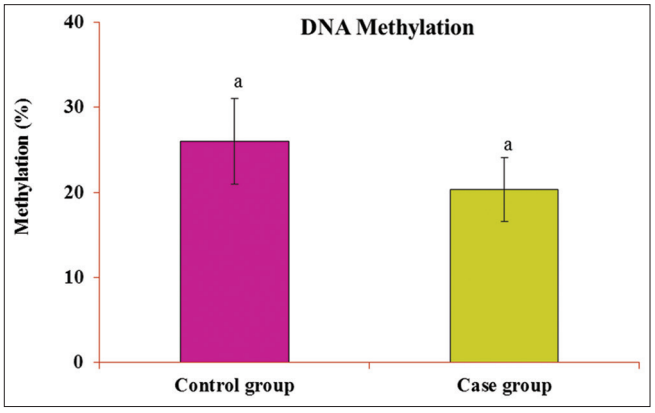

Figure 4: Percent DNA methylation in subjects included in the study (values are Mean ± SE; small letter (a) represent a comparison of means based on Tukey's post-hoc test)

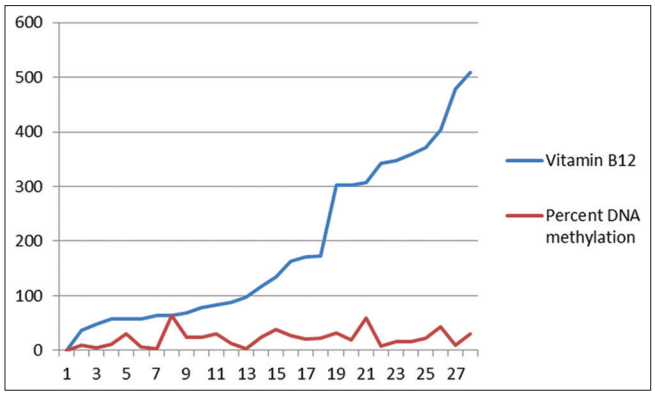

Figure 5: Correlation between serum vitamin B12 levels and percent methylation defect

and the concentration of blood haemoglobin (14.89 ± 0.34 g/dL) were recorded higher in the subjects of control group than in the case group (7.70 ± 0.87 million/mL, 101.94 ± 17.98 pg/mL, and 13.13 ± 0.25 g/dL, respectively).

However, the statistical results did not show any significant difference in the mean corpuscular volume (t = 0.56; *P* = 0.579; df = 22), the concentrations of leutinizing hormone (t = 1.69; *P* = 0.105; df = 23), follicle-stimulating hormone (t = 1.76; *P* = 0.091; df = 24), testosterone (t = 0.21; *P* = 0.837; df = 22) and the percent DNA methylation (t = 0.91; *P* = 0.376; df = 18) of the two groups.

Discussion

The study included 17 oligozoospermic infertile men and 10 healthy normozoospermic fertile controls. The study period was mostly affected by the COVID-19 pandemic with very frugal physical attendance of patients and medical services; this limited the sample size. The sperm was utilised for DNA methylation study; thus, azoospermic infertile patients were not included in the study.

The age of the study subjects was 22–42 years; the mean age was similar in both cases and controls (30.2 years vs 30.4 years). The study included subjects with all ranges of oligozoospermia (mild, moderate, and severe). Amongst the case group, the lowest sperm concentration was 2 million/mL and the highest sperm concentration was 13 million/mL. The mean value of haemoglobin in the control group was significantly higher than that in the case group (14.89 ± 0.34 vs 13.13 ± 0.25). The haemoglobin concentrations in the study participants were in line with serum vitamin B12 levels in the participants. However, the statistical results did not show any significant variation in the mean corpuscular volume (t = 0.56; *P* = 0.579; df = 22) of the two groups. For years, complete hemogram, particularly mean corpuscular volume (MCV) has been used as screening criteria for B12 deficiency. Macrocytosis [MCV >100 femtolitres (fl)] often precedes anaemia.[14] However, recent studies have unravelled that MCV might not be informative in subclinical B12 deficiency. Patel *et al*.[15] (2017) found raised MCV only in 1% of vitamin B12 deficient cases, and the sensitivity was also very low. Similarly, Bhatia *et al*.[16] found 25% macrocytes in B12 deficient subjects. A systematic review of the diagnostic value of an elevated MCV for B12 deficiency in both anaemic and nonanemic patients showed that the sensitivity of the MCV for B12 deficiency in non-anaemic individuals was 17%, whereas the sensitivity was 30% for B12 deficiency in patients with anaemia.[17] Therefore, the MCV should not be used as the only parameter ruling out the diagnosis of B12 deficiency.

Hormonal parameters such as LH, FSH, and testosterone have significant effects on spermatogenesis and sperm count. In our study, no significant difference was found in the concentration of these hormones between the two groups. Therefore, any anticipated effect on sperm count due to hormonal parameters could be eliminated.

To date, no study has directly compared vitamin B12 levels in the serum of oligozoospermic and normozoospermic patients. However, a study by Jathar *et al*.[18] (1976) compared seminal plasma vitamin B12 levels in azoospermic, oligozoospermic, and normozoospermic subjects and found that azoospermic subjects had lower levels of seminal vitamin B12 when compared to oligozoospermic and normozoospermic males. Similarly, Boxmeer *et al*.[19] (2007) demonstrated a significant positive correlation between the total cobalamin concentration in seminal plasma and the sperm concentration in men participating in an IVF or ICSI procedure. Moreover, they demonstrated that the total cobalamin concentration in serum was significantly correlated with the seminal plasma concentration. Therefore, our results are in line with the findings of Boxmeer *et al*.[19] and clearly demonstrate the association between serum vitamin B12 and low sperm count. Although no study has directly compared serum vitamin B12 levels in oligozoospermic and normozoospermic subjects, several studies have demonstrated the effect of vitamin B12 supplementation on sperm parameters (Isoyama *et al*.,[20] Iwasaki *et al*.,[21] Gual-Frau *et al*.[22]). The above evidence clearly shows the association between vitamin B12 deficiency and low sperm count. Our study also suggests the possible relationship between serum vitamin B12 levels and sperm count.

One possible mechanism, by which vitamin B12 deficiency can cause oligozoospermia is by affecting DNA methylation by participating in a process known as 'one-carbon metabolism'. When vitamin B12 concentration is low, S-Adenosyl Methionine (SAM) synthesis is reduced. As SAM is the principal methyl donor in methylation reaction, DNA methylation is theoretically reduced in the vitamin B12 deficient state.

Our study involved the comparison of DNA methylation profiles of sperm from fertile and infertile human males. Several studies (Kobayashi *et al*.,[23] 2007; Marques *et al*.,[24] 2008; Poplinski *et al*.,[25] 2010) have compared sperm DNA methylation profiles of some specific genes or regions from fertile and infertile males. These studies analysed only a

small number of genes or imprinted regions. In contrast, we compared the DNA methylation profiles of sperm from infertile males with the sperm of fertile individuals at the genome-wide level. We hypothesised that vitamin B12 deficiency causes DNA methylation defects that, in turn, result in infertility. In our study, serum vitamin B12 levels were significantly higher in fertile controls than in infertile males.

The results of our study show that oligozoospermic men have relatively lower global DNA methylation as compared to normozoospermic control; however, this difference was not statistically significant (P = 0.37). Our results are in discordance with the findings of Montjean *et al*.[26] (2015), where they showed that oligozoospermic men had significantly lower levels of global sperm DNA methylation than that of normozoospermic men. However, the results of our study are in concordance with the study by Urdinguio *et al*.[27] (2015). They analysed global DNA methylation changes between normospermic fertile and infertile patients and showed no differences in global methylation between the groups. However, they compared global DNA methylation defects between normozoospermic fertile and normozoospermic infertile men, which is in contrast to our study. The present study showed that oligozoospermic men have relatively lower global DNA methylation as compared to normozoospermic control, but this difference is not significant. Therefore, our study did not find any association between sperm count and global DNA methylation.

In the present study, serum vitamin B12 levels were significantly higher in fertile controls than in oligozoospermic males. However, our study did not find any significant difference in global DNA methylation between the two groups. The present study does not suggest any correlation between serum vitamin B12 level and percent DNA methylation (r = 0.14, P = 0.76). This may be owing to the small sample size of the study.

Another possible mechanism by which vitamin B12 deficiency can cause oligozoospermia is by homocysteine-mediated toxicity to sperm. Vitamin B12 deficiency reduces the catalytic activity of methionine synthase to synthesise methionine from homocysteine, and this low activity of methionine synthase results in the accumulation of homocysteine in the plasma, also called hyperhomocysteinemia. Hyperhomocysteinemia has been found to be associated with various health-related problems, including reproductive disorders. Ebisch *et al*.[28] demonstrated that the spermatozoa of subfertile men contained significantly higher thiol concentrations as compared with those of fertile men. The detrimental effect on embryo quality of a high homocysteine concentration in the ejaculate and in follicular fluid was intriguing and suggested that homocysteine was inversely associated with fertility outcome. An *in vitro* study revealed a significant correlation between sperm parameters such as motility and count and thiol concentrations.[29]

The results of our study showed a strong association of vitamin B12 deficiency with infertility but did not show any association of vitamin B12 deficiency with global DNA methylation defect. In the present study, oligozoospermia associated with vitamin B12 deficiency might be due to vitamin B12 deficiency-mediated high homocysteine levels. However, more experimentation is further needed to validate the present findings.

Limitations

The study period was mostly affected by the COVID-19 pandemic with very frugal physical attendance of patients and medical services, which limited the sample size.

Conclusion

This study concludes a strong association of vitamin B12 deficiency with infertility but does not show any association of vitamin B12 deficiency with global DNA methylation defect.

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Authors' contribution

DK - Conceived and collected data, NKA- conceived the research.

Financial support and sponsorship

Endocrine Society of India (ESI Grant) for conduction of study.

Conflicts of interest

There are no conflicts of interest.

Data Availability statement

The datasets generated and analyzed during the current study are not publicly available as it comprises confidential data and can lead to identification. However, the data are available from the corresponding author, Dr Dharmendra Kumar (DK), upon reasonable request and permission of the Institutional Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University.

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