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Correlation of Paternal Homocysteine Level and Sperm DNA Fragmentation in Couple with Idiopathic Recurrent Early Pregnancy Loss

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Abstract

Background: Recurrent pregnancy loss is the traumatic event for couples in the effort to obtain offsprings. Various female etiologies have been extensively studied, but more than half of remain unknown. As a result of unification of sperm and oocyte, an embryo combined with any defect during spermatogenesis will also affect the quality of embryo consequently determining the pregnancy outcomes. Routine semen analysis failed to support the evidence of influence of defective sperm in recurrent pregnancy loss. Currently, examination of sperm DNA fragmentation has been added to evaluate the quality of sperm beside the routine semen analysis. We hypothesized that high sperm DNA fragmentation plays a role in the incidence of idiopathic recurrent early pregnancy loss. Beside that, the cause of sperm DNA fragmentation are numerous and remain controversial. This study was conducted to determine the impact of paternal hyperhomocysteinemia on high sperm DNA fragmentation and incidence rates of idiophatic recurrent early pregnancy loss.

Material and methods: Fourty partners of idiopathic recurrent early pregnancy loss caes and 40 cases of control from normal male fertile population were included in this study. Blood and semen samples were collected for routine semen analysis, sperm DNA fragmentation, serum and seminal homocysteine. The results were then analyzed to determine the association between sperm and DNA fragmentation, serum homocysteine, seminal homocysteine and incidence rates of idiopathic recurrent early pregnancy loss.

Results: incidence rates of idiopathic recurrent early pregnancy loss was significantly associated with sperm DNA fragmentation ($p<0.05$) and serum homocysteine ($p<0.05$). Idiopathic recurrent pregnancy loss partners had significantly higher DFI ($p<0.05$). Hyperhomocysteinemia appears to be associated with significantly increase of sperm DNA fragmentation ($p<0.05$).

Conclusion: Paternal serum homcysteine and high sperm DNA fragmentation was significantly correlated with the incidence rates of idiopathic recurrent early pregnancy loss.

Key words : hyperhomocysteinemia, DFI, idiopathic recurrent early pregnancy loss

Introduction

Recurrent pregnancy loss is a condition different with an infertility defined by two or more failed pregnancies [1]. From all clinical recognized pregnancy, 12-15% of them will end up as a miscarriage, but only less than 5% of women will experience two consecutive miscarriage, and less than 1% experience three or more [2].

Recurrent pregnancy loss has been linked all to female etiology such as chromosomal abnormality, antiphospholipid syndrome, metabolic disorders, hormonal disorders, uterine abnormalities, maternal immune dysfunction, thrombophilias, infections, enviromental and behavioral factors [3-4] but still more than half of causes are unexplained and most of the therapeutic approach are still controversial [5].

Pregnancy is the result from fusion of sperm and oocyte. The good outcome of pregnancy is a result of growth and fertilization due to sufficient quality of sperm and oocyte. Male factors may also contribute to the failure of pregnancy, thus neccesitating further investigation. The association between poor semen quality and poor embryonic development has been extensively reported. High quality sperms correspondingly produce high quality embryos. Delayed fertilization and cleavage rates have been reported to be associated with a high percentages of abnormal morphological spermatozoa when compared with successful IVF with normal spermatozoa [6]

The frequency in which sperm defect contribute to recurrent pregnancy loss has not been well established and the relation between standard semen parameters and recurrent pregnancy loss has been a controversial subject [7]. Partners of recurrent pregnancy loss patients show significant increase in sperm

chromosomal aneuploidy, abnormal chromatin condensation, DNA fragmentation, increased apoptosis and abnormal sperm morphology compared with fertile men [8].

The etiology of sperm fragmentation is much likely male infertility, appears to be multi- factorial and maybe due to intrinsic or external factors. Intrinsic factors that may predispose to sperm DNA damage include protamine deficiency [9], mutations [10], DNA packaging defects [11], advanced paternal age [12], abortive apoptosis [13], high level of Reactive Oxygen Species (ROS) [14]. External factors such as heat [15], chemotherapeutic agents [16], radiation [17] and other gonadotoxins [18] are associated with an increase in the percentage of ejaculated spermatozoa with DNA fragmentation. Although the exact mechanism involved has not been delineated, cigarette smoking [19], genital tract inflammation [20], varicoceles [21] and hormone deficiency [22] all have been associated with increased DNA fragmentation .

Therapeutic management to treat the increase DNA damage of the sperm with antioxidant might be benefit nonetheless remain controversial and other management should be sought [23].

Elevated blood level of homocysteine has been identified to increase DNA fragmentation in nerve cell [24] and hepatic cell [25] and increase the level of ROS. There may be a similar correlation between incidence of DNA sperm fragmentation and elevated level of homocysteine in the body .

Association of sperm DNA fragmentation with serum and seminal fluid level of homocysteine has not been identified. There will be a new treatment strategy if increase homocysteine in the body cause sperm DNA fragmentation which might be corrected with folic acid, cyanocobalamin and thiamine supplementation.

This study was aimed to search for the role of male factor by testing sperm DNA fragmentation and level serum and seminal homocysteine and its correlation with idiopathic recurrent early pregnancy loss .

MATERIALS AND METHODS

Patients

Subjects were recruited from IVF and Fertility Clinic Halim Fertility Center, Division of Reproductive Endocrinology and Fertility, Department of Obstetrics & Gynaecology, Faculty of Medicine – USU, H. Adam Malik General Hospital, Medan. Couples from idiopathic recurrent early pregnancy loss and healthy fertile men who had fathered a child within a year were enrolled as cases and controls. All idiopathic recurrent early pregnancy loss cases included subjects with losses from gestational age under 10 weeks of pregnancy with unidentified cause screened with comprehensive recurrent pregnancy loss tests which comprised of detailed family, clinical, occupational, lifestyle and reproductive histories, physical examination and parental genetic, endocrinological, hematological immunological, infections and anatomical abnormalities testing. Forty Cases and forty controls who fulfilled criteria of age below 60 years old and no history of sexual dysfunction and with exclusion criteria of male who had history of high fever, smokingb of more than 5 pieces per day, exposure to gonadotoxin like insecticide, pesticide, heavy metals, exposure to any medication can compromise sperms such as sulfasalazin, chemotherapeutic agent, recreational drugs, anabolic steroid, nitrofurantoin, gentamycin, erythrhromycin, occupation in high thermal setting, exposure to radiation, consume any vitamin B6, Vitamin B 12, folic acid suplements within 3 months, infection of male reproductive organ and varicocele were selected for this study .

Blood samples were collected by venipuncture between 9 and 11 a.m to assess serum homocysteine. Semen was submitted by the patients in both groups for semen analysis, to assess DFI and seminal homocysteine.

Semen samples were collected for routine semen analysis sperma DNA Fragmentation Index (DFI) testing and homocysteine level testing. After a period of 2-5 days of intercourse abstinence, participants were then asked to masturbate and contain resulting sperm in sterile containers. Sperm should be analyzed within 2 hours on production. Routine semen analysis was performed according to WHO 2010 guidelines.

Technique of sperm DNA fragmentation test using Halosperm kit (Halotech Madrid,Spain): Semen sample was diluted to 5 -10 x 10^6/ml with culture medium (Irvine scientific ,USA). Agarose gel in eppendorf from the kit was incubated in water bath of 90 ^{0} – 100^0 C for 5 minutes, until the agarose dissolved and then 5 minutes in water bath of 37^0C.Add 25 µL of semen sample into agarose eppendorf and mixed carefully. 20 µL of the mixture were extracted and pippeted on the supercoated slide, placed on a cold surface of 4 ^{0} C in the refrigerator for 5 minutes and covered with 22 x 22 mm coverslip, then coverslip was then removed and then immersed horizontally into the previously prepared acid solution by mixing 80 µL HCl with 10 mL of distilled water for 7 minutes. The slides were then transferred horizontally into lysing solution for 25 minutes. After rinsing for 5 minutes with distilled water, the slides were dehydrated in increasing concentration of ethanol (70%,90%,100%) for 2 minutes and left it dried. Slides were stained with mix of Wright's staining solution (Merck ,Germany) and Phospate - Buffered Solution (PBS) (Merck, Germany) for 5 – 10 minutes and then washed with tap water and allow to dry. Each slide was examined under 100 x objective bright field microscope. Sperms with large halo (thickness between equal to or larger than the length of the smallest diameter of the core) and sperm with medium-sized halo (thickness between greater than 1/3 of the smallest diameter of the core and

less than the smallest diameter of the core) were classified as spermatozoa having no fragmentation. Spermatozoa with small halo (thickness between equal or smaller than 1/3 of the smallest diameter of core) and without halo were classified as spermatozoa having DNA fragmentation .

Microscopic visualisation and classification of nucleis were directly analyzed. Slides were read by two personels, the results of which were were summed and then divided by two to avoid interobserver bias. Semen and blood sample for seminal homocysteine testing was sent to Prodia Laboratory, Medan, Indonesia.

Liquefied semen samples were centrifuged at 3000 rpm for 10 minutes .The supernatant of seminal plasma was taken and transferred into eppendorf tubes .The seminal plasma was frozen at – 20^0 C until examination.

Blood samples were taken by venipuncture after overnight fasting .The sample were centrifuged at 3000 rpm for 10 minutes and serum was separated from red blood cells .The blood serum was frozen at – 20 C until examination.

Total homocysteine levels of seminal plasma and blood serum were measured using chemicalluminescence assay (competitive immunoassay) by using ADVIA Centaur (Siemen, Tarrytown, NY,USA).

Ethical clearance was obtained from the ethical committee of School of Medicine, Universitas Sumatera Utara. All participants were fully informed regarding the study and they were asked to sign the informed consent upon all informations provided.

Data analysis

Data were processesed and analyzed using SPSS 16 (Statistic Package for Social Science) software. Univariate analysis for each variable was done to look for distribution of variables. Interobserver reliability for DFI test was tested using alpha cornbach test. Differences between individual groups with numeric data were analyzed by using Student t test and mann Whitney U test and for categorical data using Chi square test. Correlations between variables were determined using pearson and spearman correlation test. A *p* value <0.05 is taken as the threshold for statistical significance. Correlation of more than 2 variables with categorical data was tested by using logistic regression.

RESULTS

Determinant factors in idiopathic recurrent early pregnancy loss

Subjects who met the inclusion and exclusion criteria of control and case groups were recruited. There were 80 patients selected for this study and divided equally between control and case group. The blood samples of these patients were collected to assess serum homocysteine. Semen was submitted by the patients in both groups for semen analysis, DFI test and seminal homocysteine test.

The mean age was 34.05 years in control group and 25.70 years in case groups. The mean Body Mass Index (BMI) was 25.27 in control group and 25.70 in case group. There were no significant different in age and BMI in both groups.

Results of semen analysis in both groups, seminal volume was found to be similar in both groups ($p \geq 0.05$), for sperm density, lower counts was found in case group compared to control group, but statistically no significant difference ($p \geq 0.05$), in sperm motility grade A, the case group had lower percentage than controls, but there was no statistically significant difference ($p \geq 0.05$), total motility (A + B) in the case group was significantly lower compared to the control group ($p < 0.05$). As for normal forms, the percentage in case group was slightly lower compared with the control group but not statistically significant ($p \geq 0.05$).

For the result of sperm DNA fragmentation, it was found that DFI in case group was significantly higher compare to control group ($p<0.05$). Serum homocysteine was significantly higher in case group ($p<0.05$). Meanwhile seminal homocysteine level was found significantly higher in control group ($p<0.05$).

Variables	Control		Case		P
	Mean ±SD	CI	Mean±SD	CI	
Age (year)	34.05±5.08	32.43-35.67	34.02±5.73	32.19-35.86	0.984[a]
BMI	25.27±3.20	24.25-26.29	25.70±4.99	24.1-27.19	0.729[b]
Semen volume (ml)	3.52±1.29	3.07-3.97	3.52±1.41	3.01-3.92	0.900[b]
Density (10^6/ml)	36.39±20.1	29.96-42.81	31.74±25.52	23.57-39.90	0.368[a]
Motility A (%)	24.50±11.87	20.70-28.30	19.75±14.54	15.1-24.40	0.136[b]
Motility (A+B) (%)	54.35±15.70	49.33-59.37	45.15±15.11	40.32-48.98	0.009[b]
Normal forms (%)	10.78±4.34	9.39-12.16	10.15±5.09	8.52-11.78	0.585[b]
DFI	16.02±8.33	3.36-18.69	34.12±16.53	28.84-39.41	0.000[a]
Serum HCY	6.72±4.76	5.19- 8.24	13.57±6.16	11.60-15.54	0.000[b]
(µmol/L)	5.77±4.31	4.40-7.15	3.26±10.03	0.052-6.47	0.000[b]
Seminal HCY					
(µmol/L)					

[a] student t test [b] Mann Whitney U test

Table 1. Comparison of characteristic and results of semen analysis, DFI, level of serum homocysteine and seminal homocysteine in control group and case group

All of the variables that were assumed to have influence in the incidence of idiopathic recurrent early pregnancy loss were included in the logistic regression test. It was found only DFI and serum homocysteine have influence in the incidence of idiopathic recurrent early pregnancy loss

Variable	B	exp(B)	P
DFI	0.108	1.114	0.001
Serum Homocysteine	0.276	1.318	0.003
Constant	-5.242	0.005	0.000

Table 2. End results of logistic regression test for identifying determinant factors towards incidence of idiopathic recurrent early pregnancy loss

There were no correlation found between DFI with semen volume ($p \geq 0.05$), density ($p \geq 0.05$), motility A ($p \geq 0.05$) and normal forms ($p \geq 0.05$). There were a significant correlation between DFI with motility A+B ($p < 0.05$), serum homocysteine ($p < 0.05$) and seminal homocysteine ($p < 0.05$) as shown in Table 3.

Variable	P	R
Semen volume	0.501	-0.076 (spearman)
Density	0.428	-0.090 (pearson)
Motility A	0.091	-0.190 (spearman)
Motility A+B	0.010	-0.285 (spearman)
Normal Forms	0.064	-0.208 (spearman)
Serum Homocysteine	0.000	0.498 (spearman)
Seminal Homocysteine	0.000	-0.532 (spearman)

Table 3. Correlation of DFI with other test results

Variable	Control	case	P	OR
	N (%)	N (%)		
DFI <30	38 (95.0%)	16(40%)	0.000	9.1
DFI ≥30	2 (5%)	24 (60%)		

Table 4. Correlation of high DFI and incidence of idiopathic recurrent early pregnancy loss

Sixty percent of case subjects had high sperm DNA fragmentation and only 5% of control group had high sperm DNA fragmentation, meanwhile 95% of subjects in control group had low sperm DNA

fragmentation and in case group only 40% subjects had low sperm DNA fragmentation. DFI ≥30 was significantly associated with idiopathic recurrent early pregnancy loss with Odd Ratio of 9.1.

Variable	Control	case	P	OR
	N (%)	N (%)		
HCY ≤12µmol/L	34 (85%)	17 (42.5%)	0.00	3.2
HCY >12µmol/L	6 (15%)	23 (57.5%)		

Table 5. Correlation of hyperhomocysteinemia (HCY ≥12µmol/L) with incidence of idiopathic recurrent early pregnancy loss

Hyperhomocysteinemia were found in 29 cases from all 80 subjects of study group. It was distributed in 23 cases among all patients with idiopathic recurrent early pregnancy loss and only 6 cases in control group. There was significant different between numbers of hyperhomocysteinemia cases in case group compare to control group with Odd Ratio of 3.2.

Variable	DFI<30	DFI≥30	p	95% CI
	n(%)	n(%)		
HCY ≤12µmol/L	41(75.9%)	10(38.5%)	0.00	0.186-0.678
HCY >12µmol/L	13(24.1%)	16(61.5%)		

Table 6. Correlation of hyperhomocysteinemia with high sperm DNA fragmentation

Hyperhomocysteinemia were found in 61.5% patients with high DFI (DFI≥30) and only 24.1% patients with low DFI (DFI<30). There was statistically different in number hyperhomocysteinemia patients between DFI <30 and DFI ≥30 group (p=0.00).

DISCUSSION

This study showed that semen volume, density, progressive motility, and sperm morphology did not differ significantly, only total sperm motility was lower in the group with high DFI (p = 0.009), suggesting sperm with high DFI negatively affected motility capcity. No parameters in semen analysis was proven to correlate with incidence rates of idiopathic recurrent early pregnancy loss. We assume that the routine semen analysis cannot be relied on for providing further information regarding the possible role of the male factor in the incidence of idiopathic recurrent early pregnancy loss and further additional sperm test is needed to search for a better tool.

This study found that DFI significantly differed between normal fertile men and men partners of idiopathic early pregnancy loss women (p = 0.000). High DFI was associated with pregnancy loss. Oocyte apparently limited the capacity to repair sperm DNA damage, if excessive, thus comprimising self repairing capacities of oocytes, and consequently defective embrios and causing abnormal embrional growth and consequently resulting in miscarriages [26].

Two studies reported significant sperm DNA fragmentation increase in partners of unexplained recurrent pregnancy loss women [27-28], while Gill Villa et al reported that DFI had no weight in recurrent pregnancy loss [29]. These studies showed conflicting results and did not show consistent results regarding the correlation of DFI and recurrent pregnancy loss.

We found that patients with DFI ≥30% have associated with pregnancy loss. This is in line with the finding that more clinical pregnancy loss in ICSI patients with DFI ≥27% [30]. The idea to made cutoff DFI ≥30% in this study came from "Georgetown Male Factor Infertility Study". Fertility datas from this study were used to establish the statistical threshold of >30% DFI for significant lack of fertility status [31].

The Odd ratio of DFI ≥30% for pregnancy loss was 9.1. This indicated a high probability of pregnancy loss if DFI ≥30%. Further study with a proper design for new cut off of DFI is needed to determine the risk of pregnancy loss, because cut off of DFI ≥30% is actually for determining fertility status but for pregnancy loss this cutoff maybe lower than 30.

This study showed that serum homocysteine was significantly associated with DFI (p = 0.000, r = 0.498). There was apparent correlation which in the end through this mechanism leads to increased incidence of recurrent pregnancy loss.

Both control and case groups reported 36.25% cases of hyperhomocysteinemia. Unfortunately, no studies have reported data concerning prevalence rates in Indonesia. Prevalence rates are expectantly higer in

western population. However cases of thermolabile variant MTHFR are not included as they are only detected by using methionine loading test. Because of different cut off set for definition of hyperhomocysteinemia as comparison of this incidence become difficults. Some of studies used cut off 15µmol/L [32] and some used cut off 11.4µmol/L for men [33].

Hyperhomocysteinemia cases was significantly more more frequently encountered in recurrent pregnancy loss patients compares to normal fertile men with Odd ratio of 3.2 indicating a high probability of recurrent pregnancy loss will occurred if homocysteine level >12µmol/L.

Number of hyperhomocysteinemia cases in DFI >30 males was significantly higher than in DFI<30 males. Hyperhomocysteinemia was associated with DNA fragmentation in nerve cell and hepatic cell and in this study showed hyperhomocysteinemia associated with sperm DNA fragmentation.

Level homocysteine in blood is not correlated with seminal homocysteine level. This study reported that seminal homocysteine levels in normal fertile male was lower than male from couple with idiopathic recurrent early pregnancy loss ($p = 0.000$, $r = -0.532$). Whether levels exceed the normal range cannot be determined, because no study has investigated normal cut off values for seminal homocysteine levels. Seminal fluid is a mixture of secretion from seminal vesicles (60% to 70%), prostatic glands (15% to 30%), litre gland, cowper glands (5%), spermatozoa and epididymal fluid (less than 10%) [34]. Therefore more than 90% of seminal plasma is derived from glands outside the testicle tissue and may have different from the liquid in the seminiferous tubules and epididymis where spermatogenesis and maturation take place. Beside that homocysteine may not passed the blood testis barrier easily, this may explain why the level of seminal homocysteine was not in positive correlation with serum homocysteine [35].

This study found lower sperm DNA fragmentation with higher level seminal homocysteine in fertile male patients compare to higher DNA fragmentation in lower level of seminal homocysteine. Higher levels of seminal homocysteine will also generates more ROS production in seminal fluid indicating DNA fragmentation induced by homocysteine was not predominatly due to ROS attack in seminal fluid but mostly due to DNA hypomethylation in genetic and epigenetic process during spermatogenesis and spermiogenesis stage. Homocysteine will cause misincorporation of uracyl instead of thymine in DNA. During the normal repair processes, when the removal of misincorporated uracyl fails, double strands break resulting in chromosomal instability.

Elevated total homocysteine levels are usually normalized by treatment with folic acid, vitamin B_6 and B_{12}. This is related to the mechanism of homocysteine metabolism. Homocysteine is formed during the methionine metabolism at the intersection of two metabolic pathways. In the reaction catalyzed by vitamin B_{12}-dependent methyl transferase, homocysteine can acquire the methyl group from methyltetrahydrofolate to regenerate methionine. Excess of homocysteine that not copt by pathway to methinonine is catabolized to cysteine by two pathways involved vitamin B_6-dependent enzymes [36].

Folate, pyridoxine, and cyanocobalamin may reduce homocysteine level in human body and may have role in declining sperm DNA fragmentation and subsequently may reduce the incidence of recurrent pregnancy loss. Further study is needed to investigate whether the folate, pyridoxine, and cyanocobalamin supplementation in male patients will reduce sperm DNA fragmentation and incidence of recurrent pregnancy loss.

In summary, paternal hyperhomocysteinemia and sperm DNA fragmentation $\geq$ 30 significantly correlated to the incidence of idiopathic early recurrent pregnancy loss.

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