Conference Paper

The Association between Sperm DNA Fragmentation and Idiopathic Early Recurrent Pregnancy Loss

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Abstract

Introduction. Various etiologies of recurrent pregnancy loss (RPL) have been extensively studied, but more than half of them still remain unknown. Male factor may play a role in incidence of idiopathic early recurrent pregnancy loss. Sperm DNA fragmentation as one of sperm test for male factor can be measured and expressed by a DNA Fragmentation Index (DFI). The aim of the study is to evaluate the association between sperm DNA fragmentation and the incidence of idiopathic early recurrent pregnancy loss. **Material and Methods.** A prospective study was done by recruiting 40 cases of male couple from patients with a history of idiopathic early recurrent pregnancy loss and 40 cases of control from normal fertile population from May 2010 to September 2011 in Halim Fertility Center. Sperm DNA fragmentation was detected by halosperm kit. **Results.** Both of groups were comparable in terms of the age of male patients, body mass index, duration of infertility, history of miscarriage and sperm parameters. Sperm DFI in the case group was 34.12%. and in the control group was 16.02%. There was significantly higher sperm DFI in the case group than in the control group. Sperm DFI < 30 was increased in control group (95%) compared with case group (40%). Sperm DFI ≥ 30 was increased in case group (60%) compared with control group (5%). There was a significant association between sperm DFI $\geq$ 30 and idiopathic early recurrent pregnancy loss ($p < 0,05$). **Conclusion.** There is an association between higher sperm DNA fragmentation and incidence of idiopathic early recurrent pregnancy loss.

Keywords: Sperm DNA fragmentation, recurrent pregnancy loss, DNA Fragmentation Index

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1. Introduction

Recurrent pregnancy loss is a condition different with an infertility defined by two or more failed pregnancy [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

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and behavioral factors [3-4] but still more than half of causes are unexplained and most of the therapeutic approach are still controversial [5].

There are contributions from male factor in the failure of pregnancy, which should be investigated further. The relationship between poor semen quality and poor embryonic development has been extensively reported. Good quality sperms yields good quality embryo. It is reported that delayed fertilization and cleavage rates were associated with high percentages of morphological abnormal 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 like to 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]. Sperm DNA fragmentation as one of sperm test for male factor can be measured and expressed by a DNA Fragmentation Index (DFI).

The aim of the study is to evaluate the association between sperm DNA fragmentation and the incidence of idiopathic early recurrent pregnancy loss.

2. Material and Methods

2.1. Patients

A prospective study was done by recruiting 40 cases of male couple from patients with a history of idiopathic early recurrent pregnancy loss and 40 cases of control from normal fertile population from May 2010 to September 2011. Subjects of the study were recruited from IVF and Fertility Clinic Halim Fertility Center, Division of Reproductive Endocrinology and Infertility, 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 were losses from gestational age below 10 weeks of pregnancy with unidentified cause screened with comprehensive recurrent pregnancy loss tests which included a 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, smoking of more than 5 pieces perday, 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. Semen was submitted by the patients in both groups for semen analysis and assessing DFI.

Semen samples were collected for routine semen analysis and sperma DNA Fragmentation Index (DFI) testing. The participants of the study were asked to produce sperm by masturbation and collected in sterile container with period of 2-5 days of intercourse abstinence. Sperm should be analysed within 2 hours after produced. 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°– 100°C for 5 minutes, until the agarose dissolved and then 5 minutes in water bath of 37°C. Add 25 μL of semen sample into agarose eppendorf and mixed carefully. Take 20 μL of the mixture and pippeted onto the supercoated slide, placed on a cold surface of 4°C in the refrigerator for 5 minutes and covered with 22 x 22 mm coverslip, then removed coverslip and immersed the slide horizontally into the previously prepared acid solution by mixing 80 μL HCl with 10 mL of distilled water for 7 minutes. Then the slide were transferred horizontally into lysing solution for 25 minutes. After rinsing 5 minutes with distilled water, the slide 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 of bright field microscope. Sperm with large halo (thickness between equal 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 the 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 the nuclei are direct visual analysis and slides were read by two personels and the results from both examiners will be summed and then divided by two to avoid interobserver bias. 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°C until examination.

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.

2.2. Data Analysis

Processing and analyzing data were using SPSS 17 (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 by 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 was using Chi square test. Correlations between variables were 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

3. Results

3.1. 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. Semen was submitted by the patients in both groups for semen analysis and DFI 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 compare 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 compare 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 ($p < 0.05$).

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.

	Control		Case		P
Variables	**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+B) (%)	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]
[a] student t test, [b] Mann Whitney U test					

Table 1: Comparative of characteristic and results of semen analysis, DFI, in control group and case group.

Variable	B	exp(B)	P
DFI	0.108	1.114	0.001
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.

It was found only DFI have influence in the 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 was 60% of subjects from case group 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.

4. Discussion

The 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 ($p = 0.009$), this suggests that sperm with high DFI have negatively effect

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)

Table 3: Correlation of DFI with other test results.

	Control	case	p	OR
Variable	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.

their ability in motility. We could not find any of parameters in semen analysis that has proven to correlate with the incidence 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.

In this study we found significant differences of DFI between normal fertile men and men from couple with idiopathic early pregnancy loss (p = 0.000). There was a correlation between high DFI with pregnancy loss. Oocyte has limited ability to repair sperm DNA damage, if the damage is too much, then the repair ability of oocytes will be not sufficient, and it will produce a defective embryo and cause the abnormal growth of embryos and fetuses which will end up as a miscarriage [24].

Two studies have found the significant increase in sperm DNA fragmentation in the couples with unexplained recurrent pregnancy loss [25-26], while Gill Villa et al found DFI had no weight in recurrent pregnancy loss [27]. 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% [28]. 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 [29].

The Odd ratio of DFI ≥ 30% for pregnancy loss was 9.1. This means that high probability of pregnancy loss if DFI ≥ 30%. Further study with 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.

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.

5. Conclusion

There is an association between higher sperm DNA fragmentation and incidence of idiopathic early recurrent pregnancy loss.

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