Comparative Study of the Effects of Tamoxifen Citrate and Folate on Semen Quality of the Infertile Male with Semen Abnormality

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Objective: *To assess the effect of treatment with a combination of the antiestrogen Tamoxifen citrate and Folate on semen quality of the infertile male with semen abnormality.*
Design: *Prospective randomized controlled trial study.*
Material and Method: *Between May 2013 and October 2014, 68 infertile male with semen abnormality were asked to join the present study. Informed consents were signed; all patients were divided into four groups, given placebo (control), Tamoxifen citrate 20 mg/day, Folate 5 mg/day, and Tamoxifen citrate plus Folate for continuous three months. The result of treatments i.e., semen parameters, hyaluronan binding assay, hypo-osmotic swelling test, and DNA damage test were evaluated at baseline, at the end of drugs treatment (3-month), and at 3-month after discontinuation of treatment (6-month).*
Results: *Tamoxifen alone caused a significant increase in sperm concentration, while Tamoxifen plus Folate significantly increased both sperm concentration and sperm motility at 3-month after treatment. Folate alone and Tamoxifen plus Folate significantly decreased DNA tail length at 3-month and at 3- and 6-month after treatment, whereas Tamoxifen alone caused no significant change in DNA tail length. Sperm DNA integrity was improved as seen by decrease in the length of DNA tail.*
Conclusion: *Our study indicated that Folate in combination with Tamoxifen citrate could improve sperm quality including semen parameters and sperm DNA integrity.*

Keywords: *Infertility, Semen quality, Tamoxifen citrate, Folate*

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Male infertility is an important factor for infertile couple and 30 to 50% of sub-fertile couples male partner with suboptimal semen quality are affected from either low sperm count, poorly motile sperm, or sperm with abnormal size, and shape (morphology)[1]. Many aspects of male factor infertility are still poorly understood, and patients are often diagnosed with idiopathic oligoteratoasthenozoospermia (OAT). For this reason, the management of male infertility is classified into etiological or empirical treatments[2]. However, now, suboptimal semen quality has no definite treatment.

Tamoxifen, a non-steroidal antiestrogen, represents a significant advance in the treatment of female breast cancer and was introduced three decades ago as empirical treatment for idiopathic oligozoospermia[3]. World Health Organization Working Committee has proposed Tamoxifen citrate as the first line of treatment for idiopathic oligozoospermia[4].

Folate is a water-soluble vitamin B that is naturally present in some foods, added to multiple vitamins, and available as a dietary supplement. Folate is now viewed not only as a nutrient needed to prevent megaloblastic anemia in pregnancy but also as a vitamin essential for reproductive health[5]. Adequate Folate intake is vital for cell division and homeostasis due to the essential role of Folate coenzymes in nucleic acid synthesis, methionine regeneration, and in the shuttling, oxidation, and reduction of carbon, required of normal metabolism and regulation[6]. Because DNA synthesis is the main part of spermatogenesis, Folate is also probably important to this process. From the study of Boxmeer et al[7], they found that low Folate concentration in the seminal plasma of a subgroup of fertile men was associated with increased levels of sperm DNA damage.

Previous studies have been conducted using Tamoxifen with other drug to improve the semen

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quality. From the report of Adamopoulos et al[8-11], the effects of treatment with Testosterone in combination with Tamoxifen citrate improved sperm variables and increased pregnancy rates in men with idiopathic oligozoospermia. It is not certain whether the other combination regimens could improve semen quality in the infertile men with abnormal semen quality. However, our previous study reported that Testosterone combined with Folate could improve semen parameter including sperm DNA integrity[12].

The objective of the present study, performed as a double-blind, randomized control trial, was to evaluate the effect of treatment with a combination of the antiestrogen Tamoxifen citrate and Folate on semen quality i.e., semen parameters, hyaluronan binding assay, hypo-osmotic swelling test, and DNA damage test by Comet assay in infertile male with semen abnormality.

Material and Method

Between May 2013 and October 2014, we enrolled 68 men with abnormal semen analysis who attended the Infertility Clinic of Thammasat University Hospital, Thailand. After the introduction and discussion concerning the study, they were asked to sign the informed consent form. Inclusion criteria included abnormal semen analysis of at least one parameter according to World Health Organization Criteria 2010[13] (concentration <15 million/ml, motility <40%, or morphology <4%), failure of the female partner to conceive after one year of regular unprotected sexual intercourse, no history of Tamoxifen and Folate allergy. Overall exclusion criteria were the followings, use of Tamoxifen and Folate within three months before recruitment, use of other medicines or vitamin during study period. Then, the 68 subfertile men were randomized into four groups, group 1 (control group), patients in this group were prescribed placebo tablet, group 2, Tamoxifen citrate treatment alone, patients received Tamoxifen citrate at a dose of 20 mg one time per day, group 3, Folate treatment alone, in which Folate was given at a dose of 5 mg per day, and group 4, combined Tamoxifen citrate and Folate treatment, in which Tamoxifen citrate and Folate were given as in group 2 and 3. Treatments were carried on for a period of three months and then the outcomes of treatment were evaluated three and six months after treatment. Data collections included patient's age, body mass index, smoking, alcohol drinking, and the infertility assessment i.e., hormone assays (follicle-stimulating hormone (FSH), luteinizing hormone (LH), and Testosterone), semen parameters (sperm concentration, sperm motility, and sperm morphology), hyaluronan binding assay, hypo-osmotic swelling test, and DNA damage test by Comet assay were collected at baseline, three, and six months after treatment. Ethical approval of the present study was obtained from the Research Ethical Committee No. 1, the Faculty of Medicine, Thammasat University.

Measurements of outcomes

Hormone assays

Hormone assays (FSH, LH, and Testosterone) were determined by Microparticle Enzyme Immunoassay (MEIA) and Chemiluminescence methods. Blood samples were collected in the morning and sent to laboratory within 30 minutes.

Semen analysis

Semen samples were collected by masturbation from infertile men after two to five days of recommended abstinence. After liquefaction, routine semen analysis was performed according to WHO guidelines[13]. Sperm concentration and motility was evaluated by Computer Assisted Sperm Analysis (CASA; IVOS, Hamilton, version 10), sperm morphology was identified via Papanicolaou (PAP) staining and viability of sperm was assessed using hypo-osmotic swelling test (HOS)[14].

Hyaluronan binding assay (HBA)

The procedure was performed following the manufacturer's instructions (Origio, Inc., 2011) using a HBA kit slide (Biocoat, Inc., PA, USA); the HBA was performed immediately after sperm preparation. The prepared semen was gently mixed and 10 µL of it pipetted onto the center of the HBA slide, covered with a cell-Vu gridded cover slip and incubated at 37°C in a 5% CO_2 incubating chamber for 10 minutes. The spermatozoa were evaluated under a phase contrast microscope 400x magnification, counted twice by two embryologists blinded from each other, and the average percent bound recorded. If less than 30 motile spermatozoa were present within the counting area, a second HBA slide and the same sperm sample were analyzed once again. Bound sperm demonstrates vigorous tail beating with no progressive movement while unbound sperm swims freely. The predominant class (bound or unbound) of motile sperm was counted first (at least 100 spermatozoa). Immediately, the count was repeated in exactly the same number of grid squares, counting the other class of motile sperm. If

the total spermatozoa were less than 100, counting in 100 grid squares was performed. The percentage of HA-bound spermatozoa was calculated by dividing the bound motile spermatozoa by the total of bound and unbound motile spermatozoa and then multiplied by 100.

DNA damage test

Sperm DNA fragmentation was assessed by Comet assay[15]. The Comet assay is a single-cell gel electrophoretic assay that quantifies broken strands of DNA in individual spermatozoon. The Comet assay was performed as described by Singh et al[16]. Briefly, 30 µl of cell suspension (≈400,000 cells/ml) were mixed with 70 µl of 1% low-melting point (LMP) agarose and added to fully frosted slides that had been covered with a bottom layer of 1% normal melting point (NMP) agarose. The slides were immersed in cold lysing solution (2.5M NaOH, pH 10, 0.1M EDTA, 0.01M Tris, and 1% Triton X-100) and the cells were lysed for 1 hour at 4°C. The slides were then placed in the electrophoresis solution (300 mM NaOH, pH 13, 1 mM EDTA,) for 20 minutes to allow DNA unwinding, and electrophoresed for 20 minutes at 25V and 300 mA. After electrophoresis, the slides were neutralized in 0.4M Tris buffer (pH 7.5), stained with ethidium bromide (5µg/ml) and analyzed using a fluorescence microscope (Olympus BX-50). Images of 50 randomly selected cells (25 per replicate slide) per experimental point were analyzed with image analysis software (VisCOMET, TillPhotonics, Germany). The length of the comet tail correlates with DNA damage and the length of DNA tail was used as the measure of DNA damage.

Statistical analysis

Data were analyzed using SPSS version 22.0 software. Data outcomes were presented as mean ± SE. Analysis of variance (ANOVA) was used for comparison of baseline mean semen parameters and baseline mean hormonal levels among four groups. Comparison of mean values of pretreatment (baseline), three months, and six months after treatment groups and comparisons of mean values between groups was done using student t-test and $p<0.05$ was considered as significant.

Results

Sixty-eight men with abnormal semen analysis according to WHO criteria[13] were enrolled in the present study. Eight patients were excluded from the study (three patients declined to participate and five patients stop medication before completing the trial). Of the 60 recruited patients, 15 subjects were randomized in four groups. No statistically significant differences were found between the four groups in age, body mass index, alcohol drinking, and smoking (Table 1).

Baseline hormone concentrations were within normal range and no statistically significant differences were found between the four groups (Table 1). In

Table 1. Baseline characteristics of the participants

Characteristic	Control group	Tamoxifen citrate	Folate	Tamoxifen citrate and Folate	*p*-value
Age (years)	36.80±1.62	35.53±1.55	35.93±1.35	36.00±1.24	0.940
Body mass index (kg/m^2)	24.71±0.84	23.90±1.11	26.08±0.76	25.29±0.85	0.385
Smoking					
Yes	2	3	5	1	0.27
No	13	12	10	14	
Alcohol drinking					
Yes	6	6	4	4	0.75
No	9	9	11	11	
Semen parameters before treatment (baseline)					
Concentration (million/ml)	92.57±13.99	65.46±6.80	61.50±6.02	76.56±11.74	0.14
Motility (%)	14.33±2.44	13.06±2.47	11.40±2.41	16.86±2.61	0.46
Morphology (%)	2.20±0.48	1.86±0.42	2.06±0.70	1.86±0.60	0.96
Basal hormonal levels concentration					
FSH (mIU/ml)	4.16±0.52	4.78±0.45	4.66±0.58	5.08±0.77	0.74
LH (mIU/ml)	3.96±0.54	4.30±0.54	4.35±0.36	3.72±0.37	0.75
Testosterone (ng/dl)	373.60±30.15	339.86±38.07	341.06±28.90	402.53±39.44	0.52

FSH = follicle-stimulating hormone; LH = luteinizing hormone

Tamoxifen group, FSH, LH, and Testosterone levels increased significantly at 3-month after treatment (4.78±0.45, 4.30±0.54, and 339.86±38.07 vs. 6.66±0.76, 5.88±0.56, and 684.26±60.92 respectively), and Testosterone increased significantly after six months of treatment (339.86±38.07 vs. 429.13±32.38). In Tamoxifen plus Folate groups, FSH, LH, and Testosterone levels also increased significantly at 3-month after treatment (5.08±0.77, 3.72±0.37, and 402.53±39.44 vs. 8.16±1.34, 6.97±0.92, and 719.66±74.04 respectively). For groups comparisons, at 3-month after treatment, mean FSH, LH, and Testosterone were statistically significant increased in Tamoxifen and Tamoxifen plus Folate groups (4.34±0.63, 3.98±0.62, 380.80±33.11 in control group vs. 6.66±0.76, 5.88±0.56, 684.26±60.92 in Tamoxifen group, and 8.16±0.34, 6.97±0.92, 719.66±74.04 in Tamoxifen plus Folate group respectively) (Table 2).

There was no difference in baseline sperm parameters (Table 1). Sperm concentration markedly and significantly improved at 3-month after treatment both in Tamoxifen and Tamoxifen plus Folate groups (in Tamoxifen group, mean sperm concentration at baseline was 65.46±6.80 compared to 86.22±9.46 at 3-month after treatment, and in Tamoxifen plus Folate group, mean sperm concentration at baseline was 76.56±11.74 compared to 113.88±16.02 at 3-month after treatment). Sperm motility was increased significantly at 3-month after treatment in Folate and Tamoxifen plus Folate groups (in Folate group, mean sperm motility baseline was 11.40±2.41 compared to 20.40±3.97 at 3-month after treatment and in Tamoxifen plus Folate group, mean sperm motility baseline was 16.86±2.61 compared to 25.36±3.87 at 3-month after treatment), whereas sperm morphology was not statistically significant changed in all after treatment groups (Table 3).

The viability of sperm (HOS) was not statistically significant different in all after treatment groups. In addition, the sperm function test (HBA) was not different in all after treatment groups. However, when compared to the control group, there was statistically significant increase of HBA in Folate and Tamoxifen plus Folate groups at 3-month after treatment (41.73±6.90 in control group vs. 66.08±6.44 in Folate group and vs. 58.32±5.14 in Tamoxifen plus Folate group) (Table 4).

DNA damage measured by the DNA tail length was decreased significantly in Folate group at 3-month after treatment (in Folate group, mean DNA tail length baseline, 14.59±5.15 vs. 4.04±0.94 at 3-month after treatment). In Tamoxifen plus Folate group, mean DNA tail length was decreased at 3- and at 6-month after treatment (baseline, 21.02±5.07 vs. 5.63±1.51 vs. 6.93±2.00 at 3-month and 6-month after treatment values, respectively) (Table 4).

Discussion

There were several trials of Tamoxifen citrate and Folate treatment in oligozoospermic patients. A previous study conducted by Adamopoulos et al assessed the effect of combined Tamoxifen citrate and Testosterone undecanoate treatment on seminal parameters in men with idiopathic oligozoospermia and found that total sperm number was markedly different from pretreatment in both Tamoxifen citrate and Tamoxifen citrate with Testosterone undecanoate groups, and the percentage of good progressive motility was increased markedly in Tamoxifen citrate with Testosterone undecanoate group[10]. Similar to our study, semen parameters (concentration and motility) increased significantly in Tamoxifen and Tamoxifen plus Folate groups. Tamoxifen is believed to enhance spermatogenesis by increasing FSH and Testosterone level[17,18]. In our study, FSH, LH, and Testosterone were increased in Tamoxifen and Tamoxifen plus Folate group indicating normal pituitary function in reproduction. Wong et al reported that sperm counts increased after 26 weeks of supplementation with both Folic acid (5 mg/day) and zinc (66 mg/day), but not after supplementation with Folic acid or zinc alone in fertile and subfertile men[19]. Unlike Wong et al study, our study had shown sperm concentration was not improved after Folate treatment, whereas sperm motility increased. Swayne et al demonstrated that Folate deficiency in adult mice caused a decrease in sperm number, and increased in germline chromatin damage and DNA mutation[20]. Moreover, Boxmeer et al also reported that low Folate in seminal plasma was associated with increased sperm DNA damage[7]. Our previous study had shown that Folate and Testosterone undecanoate combined could improve DNA integrity[12]. As well as in the present study, mean DNA tail length after three and six months treatment was decreased in Folate and Tamoxifen plus Folate groups indicating decreased DNA damage in these groups of this study.

In our study, we combined Tamoxifen and Folate to determine synergistic effect to improve both semen parameters and DNA integrity in the infertile men with abnormal semen quality, and our results showed that the combination of Tamoxifen with Folate produced a satisfactory response in sperm quality. In

Table 2. Comparisons of hormonal levels (FSH, LH, and Testosterone) between control group and three treatment groups of before, after complete 3 months of treatment completed and 3 months after discontinuation of treatment

Group	FSH (mIU/ml)			LH (mIU/ml)			Testosterone (ng/dl)		
	Baseline	3-month	6-month	Baseline	3-month	6-month	Baseline	3-month	6-month
Control	4.16±0.52	4.34±0.63	3.98±0.55	3.96±0.54	3.98±0.62	3.54±0.38	373.60±30.15	380.80±33.11	362.40±34.94
Tamoxifen	4.78±0.45	6.66±0.76*,**	4.40±0.34	4.30±0.54	5.88±0.56*,**	4.48±0.46	339.86±38.07	684.26±60.92*,**	429.13±32.38*
Folate	4.66±0.58	4.57±0.59	4.54±0.51	4.35±0.36	4.26±0.38	5.16±0.41**	341.06±28.90	373.46±33.01	360.26±29.08
Tamoxifen and Folate	5.08±0.77	8.16±1.34*,**	5.01±0.68	3.72±0.37	6.97±0.92*,**	4.52±0.69	402.53±39.44	719.66±74.04*,**	434.66±42.96

* Significantly different from pretreatment (p-value <0.05)
** Significantly different from control group (p-value <0.05)

Table 3. Comparisons of semen parameters (concentration, motility and morphology) between control group and three treatment groups of before, after 3 months of treatment completed and after 3 months discontinuation of treatment

Group	Concentration (million/ml)			Motility (%)			Morphology (%)		
	Baseline	3-month	6-month	Baseline	3-month	6-month	Baseline	3-month	6-month
Control	92.57±13.99	76.24±13.10	76.13±18.29	14.33±2.44	18.06±3.46	17.33±4.28	2.20±0.48	1.60±0.51	1.40±0.37
Tamoxifen	65.46±6.80	86.22±9.46*	53.89±9.48	13.06±2.47	16.20±3.07	19.60±5.06	1.86±0.42	1.46±0.37	2.40±0.63
Folate	61.50±6.02	66.58±7.70	53.30±5.88	11.40±2.41	20.40±3.97*	15.00±2.61	2.06±0.70	1.46±0.40	1.26±0.46
Tamoxifen and Folate	76.56±11.74	113.88±16.02*	74.15±13.29	16.86±2.61	25.36±3.87*	21.20±4.72	1.86±0.60	2.40±0.74	1.93±0.45

* Significantly different from pretreatment (p-value <0.05)

Table 4. Comparisons of semen viability (hypo-osmotic swelling test, HOS), hyaluronan binding assay (HBA), and DNA damage (COMET test) between control group and three treatment groups of before, after 3 months of treatment completed and after 3 months discontinuation of treatment

Group	HOS (%)			HBA (%)			DNA tail length (μm)		
	Baseline	3-month	6-month	Baseline	3-month	6-month	Baseline	3-month	6-month
Control	44.62±5.16	48.60±5.95	44.32±5.71	45.11±8.14	41.73±6.90	40.6±7.03	11.16±4.14	10.08±3.39	8.69±4.28
Tamoxifen	42.37±6.77	51.29±5.39	48.35±5.30	49.68±7.77	59.37±7.36	47.12±6.33	8.29±1.96	6.07±1.31	5.65±1.21
Folate	38.54±6.27	50.33±5.07	46.50±4.71	54.55±8.68	66.08±6.44**	54.98±6.70	14.59±5.15	4.04±0.94*	6.01±1.49
Tamoxifen and Folate	44.97±5.03	53.16±5.23	53.83±5.17	55.72±5.99	58.32±5.14**	47.81±5.20	21.22±5.07	5.63±1.51*	6.93±2.00*

* Significantly difference from pretreatment (p-value <0.05)
** Significantly difference from control group (p-value <0.05)

Tamoxifen plus Folate group, sperm concentration and motility were significantly improved at 3-month after treatment, while in Tamoxifen group alone only sperm concentration was improved at 3-month after treatment. In the Folate group alone, only motility was significantly improved at 3-month after treatment. On the other hand, at 3-month after treatment, the percentage of HBA in Folate and Tamoxifen plus Folate groups were significantly improved compared to the placebo group. However, HOS was not significantly changed from pre-treatment. DNA tail length was decreased in Folate group at 3-month after treatment, and decreased in Tamoxifen citrate plus Folate group at 3- and 6-month after treatment, indicating decreased DNA damage in this group.

A strength of our study was that we evaluated multiple outcomes (semen parameters, concentration, motility, and morphology), hormonal changes (FSH, LH, and Testosterone), sperm viability (HOS), and sperm functions (HBA and DNA damage) that reflected to actual semen quality and integrity. However, treatment period of three months with three months follow-up covers only two spermatogenic cycles, of which one may occur while on the treatment and the second may extend over the three months after cessation of medication. Therefore, further trials on this combination should be conducted with a larger sample size and extended treatment and after treatment periods to determine and confirm the significance of the study.

What is already known on this topic?

Problems of infertility of couples are increasing and 40% of the problems are caused by male factors. Although sex hormones and vitamins have been used to treat male infertility, the results are still controversial.

What this study adds?

This report showed that Folate in combination with Tamoxifen citrate could improve sperm quality including semen parameters and sperm DNA integrity. Therefore, this combination may be use for treatment in the infertile male with abnormal semen parameters.

Potential conflicts of interest

None.

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การศึกษาเปรียบเทียบผลของ ทาม็อกซิเฟน ซิเตรท และโฟเลท ต่อคุณภาพน้ำเชื้ออสุจิของคู่สมรสฝ่ายชายที่มีบุตรยากและมีความผิดปกติของน้ำเชื้ออสุจิ

อาทิตย์ บุญยรางกูร, นิพัทธา วินะยานุวัติคุณ, เจริญไชย เจียมจรรยา, พัชรา วิสุตกุล

วัตถุประสงค์: *เพื่อศึกษาผลของการให้ยา ทาม็อกซิเฟน ซิเตรท และโฟเลท ในรูปแบบยาร่วมว่ามีประสิทธิภาพในการเพิ่มคุณภาพอสุจิให้มีคุณภาพดีขึ้นได้หรือไม่ ในกลุ่มที่ผู้มีบุตรยากฝ่ายชายที่มีความผิดปกติของน้ำเชื้ออสุจิ*

วัสดุและวิธีการ: *ทำการศึกษาระหว่างเดือนพฤษภาคม พ.ศ. 2556 ถึง ตุลาคม พ.ศ. 2557 อาสาสมัครชาย 68 ราย ที่มีปัญหาการมีบุตรยาก ถูกแบ่งเป็น 4 กลุ่ม ได้รับยาดังต่อไปนี้ กลุ่มควบคุม ยาหลอก (placebo) กลุ่มที่ได้รับทาม็อกซิเฟน ซิเตรท (Tamoxifen citrate) 20 มิลลิกรัมต่อวัน กลุ่มที่ได้รับ โฟเลท (Folate) 5 มิลลิกรัมต่อวัน และกลุ่มได้รับทาม็อกซิเฟน ซิเตรท ร่วมกับโฟเลท โดยประเมินผลการศึกษาที่ก่อนได้รับยา (baseline) หลังได้รับยาครบ 3 เดือน และหลังหยุดรับยาครบ 3 เดือน (การทดลองครบ 6 เดือน) ด้วยการเก็บน้ำอสุจิเพื่อตรวจวิเคราะห์คุณภาพอสุจิ ความมีชีวิตของอสุจิ hyaluronan binding assay ความสมบูรณ์ของดีเอ็นเออสุจิ และเจาะเลือดเพื่อวัดระดับฮอร์โมน FSH, LH และ testosterone*

ผลการศึกษา: *กลุ่มที่ได้รับทาม็อกซิเฟนอย่างเดียวมีความเข้มข้นของอสุจิเพิ่มขึ้น ในขณะที่ทาม็อกซิเฟนร่วมกับโฟเลทมีการเพิ่มขึ้นของทั้งความเข้มข้นและการเคลื่อนไหวของอสุจิอย่างมีนัยสำคัญ หลังได้รับยาครบ 3 เดือน กลุ่มที่ได้รับโฟเลทอย่างเดียวพบว่ามีการลดลงของความยาวหางของดีเอ็นเอหลังได้รับยาครบ 3 เดือน ส่วนกลุ่มที่ได้รับทาม็อกซิเฟนร่วมกับโฟเลทพบว่ามีการลดลงของความยาวหางของดีเอ็นเอทั้งหลังได้รับยาครบ 3 เดือน และหลังหยุดรับยาครบ 3 เดือน (การทดลองครบ 6 เดือน) ซึ่งการลดลงของความยาวหางของดีเอ็นเอนี้แสดงให้เห็นถึงการเพิ่มขึ้นของความสมบูรณ์ของดีเอ็นเอ*

สรุป: *พบว่าการให้ทาม็อกซิเฟนซิเตรทร่วมกับโฟเลท มีประสิทธิภาพในการเพิ่มคุณภาพของอสุจิรวมทั้งคุณภาพของดีเอ็นเออสุจิ*