Original Article

The Effect of Different Timing after Ejaculation on Sperm Motility and Viability in Semen Analysis at Room Temperature

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Objective: To determine the time variated effect on the motility, viability, and morphology parameters of sperm after ejaculation (30, 60, 120, 180, and 240 minutes).

Materials and Methods: The present study was an observational study that included 98 semen samples. Semen samples were kept in room temperature (25°C to 26°C) and were analyzed for sperm motility by computer aided sperm analysis [CASA] system and sperm viability by eosin test at 30, 60,120, 180, and 240 minutes and sperm morphology by Diff-Quick rapid test at 30 and 180 minutes.

Results: Ninety-eight semen samples were collected from 70 men. Mean age was 34.6±6.6 years old, the average BMI was 24.1±2.9 kg/m^2. Mean of sperm motility at 30, 60, 120, 180, and 240 minutes were 64.14±14.40%, 59.57±16.44%, 57.46±16.50%, 54.63±18.18%, and 54.60±18.52%, respectively. Sperm motility significantly decreased after keeping in room temperature for 60 minutes after ejaculation (p-value <0.04). Mean of sperm viability at 30, 60, 120, 180, and 240 minutes were 67.59±13.20%, 65.88±12.33%, 62.10±11%, 58.60±14.08%, and 54.10±10.37%, respectively. Sperm viability significantly decreased after keeping in room temperature for 120 minutes after ejaculation (p-value <0.001).

Conclusion: The motion parameters of sperm begin to decline in movement after 60 minutes, whereas velocity, and viability of sperm is affected after 120 minutes. Therefore, for effective and accurate semen analyses, the semen samples should be tested within one hour after ejaculation and should be stored at room temperature.

Keywords: Sperm motility, Sperm viability, Sperm velocity, Semen analysis, Time variated analysis

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Infertility is an important problem for many couples. Approximately 15% of couples in reproductive ages in the United States are affected[1]. This percentage was nearly the same as in Thailand based on a survey in 1998[2]. The number of male infertility gradually increases over time. In the United States, male infertility was found to be approximately 20% to 35% of overall of infertility couple[3]. Semen analysis was the only effective tool to find the cause of male infertility problem[4]. The analysis consists of sperm count, sperm morphology, sperm motility and viability, and secretion of accessory organ assessment[5]. Currently, infertility doctors use the Standard Guideline of the Fifth Edition of the World Health Organization [WHO] Manual[6]. The semen analysis can be measured by conventional technique or computer aided sperm analysis [CASA]. The CASA refer to a computer assisted system that is designed to provide accurate, precise, and significant information of sperm parameters. The most important aspect is to analyze the sperm motion[7].

The sperm motility is the most important parameter in evaluating the fertilization ability of the ejaculation[8-11]. In Intrauterine insemination [IUI] or in vitro fertilization [IVF], the morphology and motility of sperm are significant factors in predicting fertilization rate and pregnancy rate[12]. The original WHO guideline stated that semen sample be handled at temperature range between 20°C to 40°C while the current guideline recommends 37°C[6]. However, the data regarding of differences in optimized temperature had inconclusive results[13-16]. Aware of sperm motility's predictive power and suitable temperature range for carrying out experiments, the present study aimed to

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examine the effect of the various length of time after sperm ejaculation on motility and viability of sperm at laboratory room temperature (25°C to 26°C).

Materials and Methods

The present study was a prospective, observational study, enrolled ninety-eight healthy male partners of the infertile couples who came to Infertility Clinic, Faculty of Medicine, Ramathibodi Hospital, Mahidol University for semen analysis between February and April 2015. The semen sample will be excluded if it consisted of oligozoospermia, cryptospermia, azoospermia, or semen volume was less than 1.5 mL, its motility was less than 32%, or the total sperm count was less than 39 million/ejaculation. The abstinence period was between two and seven days before collection to prevent potential confounders and bias. The semen was collected by masturbation and ejaculation into a clean plastic container. After semen was allowed to liquefy for thirty minutes, the samples were analyzed immediately according to WHO guideline 2010.

The specimen sample was placed into one to two chambers of the CASA systems (Hamilton Thorn Research, version 10.7, Beverly, MA, USA). A 10 µl drop of sample was loaded into a 10 µm deep Makler chamber (Sefi-Mahidol Instruments, Haifa, Israel) and placed on the pre-warmed stage (29°C to 30°C) of the CASA systems. The CASA systems settings were made according to manufacturer's standard settings, frame acquired 30, frame rate 60 Hz, minimum contrast 80, minimum cell size 3 pixels, static head size 0.60 to 1.40, and magnification 1.95.

The outcome of sperm motility was defined as normal when the sperm moving with rapid and slow progression with an average path velocity [VAP] greater than 25 µm/second, VAP 5 to 25 µm/second. Each sample were examined on five replicate fields, and for the total fields summarized captured images containing more than 500 sperm and ideally more than 1,000 sperm per sample. The semen samples were kept at 25°C to 26°C (laboratory room temperature) and evaluated again by CASA at 30, 60, 120, 180, and 240 minutes after ejaculation.

The outcome of viability of sperm analysis was measured by Eosin test exclusion. The semen sample was mixed well and an aliquot of 5 µl of semen was removed and combined with 5µl of eosin solution on a microscope slide and mixed with a pipette tip, swirling the sample on the slide. The slide was covered with a 22x22 mm cover slip and left for 30 seconds. A total of 100 sperm were then counted under 40x magnification.

The sperm morphology outcome was assessed by using Diff-Quik rapid stain at 30 and 180 minutes and the viability by using Eosin test. A smear of semen was prepared on a slide. The slide was air-dried, fixed, and stained with Diff-Quik rapid stain. Fixative reagent (triarylmethane dye dissolved in methanol) was applied for 10 seconds then the slide was washed in water. Staining solution 1 (eosinophilic xanthene) was applied for 10 seconds then the slide was washed in water. Staining solution 2 (basophilic thiazine) was applied for 10 seconds then the slide was washed in water. The slide was examined with bright field optics at 1,000x magnification with oil immersion. Assessment of approximately 200 spermatozoa per replicate was made for percentage of normal forms or of normal and abnormal forms. A flow chart of the protocol is shown in Figure 1.

The present research was approved by the Institutional Review Board [IRB] in human subject studies (IRB No.02-58-49). The data were collected, encrypted, and managed at the Infertile Unit, Faculty of Medicine, Ramathibodi Hospital. The descriptive data analyses were presented as the mean standard deviation [SD] in continuous data and as the percentage in categorical data. One factor was measured in a mixed effect model by comparing different timing to sperm parameters at 30, 60, 120, 180, and 240 minutes after ejaculation. A *p*-value of less than 0.05 was considered as statistically significant. All of data were analyzed

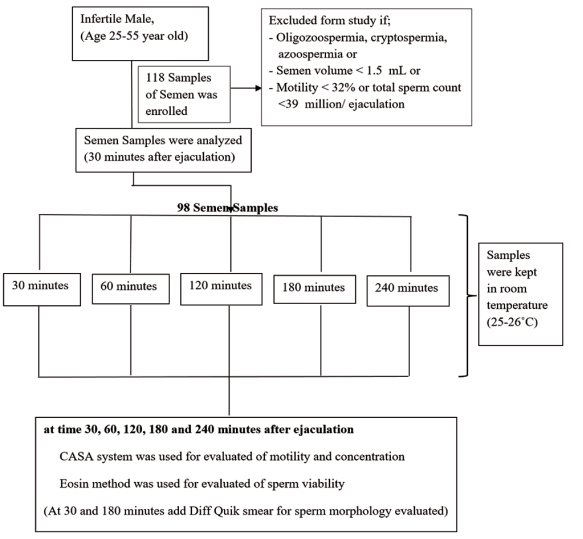


Figure 1. Flow chart.

using STATA version 13.1 (StataCorp LP, College Station, Texas, USA).

Results

Ninety-eight of 118 eligible semen samples from male partners of the infertile couples were collected and analyzed. The other 20 samples were excluded by exclusion criteria. The mean ± SD of abstinence time, time of liquefaction, pH, volume, the percentage of motility, and the percentage of normal morphology for all fresh samples at 30 minutes after ejaculation were 3.6±1.6 days, 10.13±3.52 minutes, 7.59±0.04, 2.16± 0.62 mL, 64.14±14.41%, and 1.5±0.80%, respectively (Table 1).

The mean of sperm motility at 30, 60, 120, 180, and 240 minutes after ejaculation were 64.14±14.40%, 59.57±16.44%, 57.46±16.50%, 54.63±18.18%, and 54.60±18.52%, respectively (Table 2). The means of rapid progression of sperm at 30, 60, 120, 180, and 240 minutes were (ONE NUMBER IS MISSING) 50.43±16.96%, 47.33±16.85%, 43.60±18.875%, and 43.83±18.39%, respectively. When the authors compared the different time with the 30 minutes after ejaculation, the present data showed a significant reduction of motility and rapid progression that began at 60 minutes after ejaculation by mixed effect model analysis as 64.14±14.40% versus 59.57±16.44%, *p*-value 0.004 and 54.68±17.27% versus 50.43±16.96%, *p*-value 0.005 (Table 2, Figure 2). The motility parameters of sperms were assessed by the CASA. The results showed all of the motility parameters were significantly different over time. The motility parameters demonstrated how sperm velocity was affected at laboratory room temperature after ejaculation. Curvilinear velocity [VCL] and VAP significantly decreased at 120 minutes (76.78±17.97 µm/second versus 73.26±13.71 µm/second, *p*-value 0.007 and 47.24±9.55 µm/second versus 45.07±8.10 µm/second, *p*-value 0.003, respectively). But straight-line velocity [VSL] decreased significantly after 180 minutes (35.48±6.89 µm/second versus 32.97±6.31 µm/second, *p*-value <0.001) when compared with 30 minutes (Table 3).

The means percent of sperm viability at 30, 60, 120, 180, and 240 minutes were 67.59±13.20%, 65.88± 12.33%, 62.10±11.00%, 58.60±14.08%, and 54.10± 10.27%, respectively. Interestingly, the viability started to exhibit significant reduction at 120 minutes after the ejaculation (67.59±13.20% versus 62.10±11.00%, *p*-value <0.001) (Table 2, Figure 2). The sperm's head defect and morphology significantly changed at 180 minutes when compared to 30 minutes (55.24±7.81% versus 53.91±7.85%, *p*-value <0.03 and 1.73±0.96% versus 1.52±0.80%, *p*-value <0.01, respectively) (Table 4).

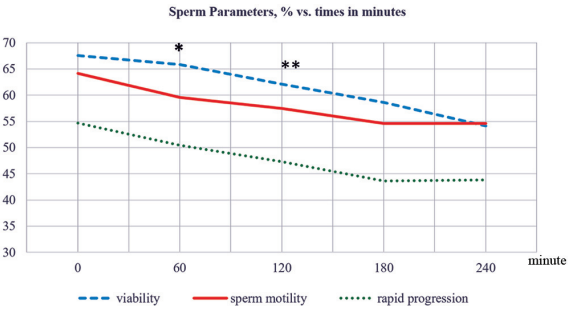
Table 1. Semen characteristics at 30 minutes after ejaculation (n = 98)

Semen parameter	Mean ± SD
Abstinence time (days)	3.60±1.60
Liquefaction (minutes)	10.13±3.52
pH	7.59±0.04
Volume (mL)	2.16±0.62
Sperm concentration (million/mL)	116.30±5.53
Percent motility (%)	64.14±14.41
Normal Morphology (%)	1.50±0.80

Table 2. Mean of percent sperm motility, rapid progression, and viability at 30, 60, 120, 180, and 240 minutes after ejaculation (n = 98)

Parameter	Time (minutes)	Mean ± SD (%)	*p*-value
Sperm motility	30	64.14±14.40	-
	60	59.57±16.44	0.004*
	120	57.46±16.50	<0.001*
	180	54.63±18.18	<0.001*
	240	54.60±18.52	<0.001*
Rapid progression	30	54.68±17.27	-
	60	50.43±16.96	0.005
	120	47.33±16.85	<0.001*
	180	43.60±18.87	<0.001*
	240	43.83±18.39	<0.001*
Viability	30	67.59±13.20	-
	60	65.88±12.33	0.165
	120	62.10±11.00	<0.001*
	180	58.60±14.08	<0.001*
	240	54.10±10.27	<0.001*

* Statistical significant if *p*-value <0.05



* Significant decrease of sperm motility and rapid progression at 60 minutes after ejaculation compared with 30 minutes (*p*-value <0.05)

** Significant decrease of sperm viability at 120 minutes after ejaculation compared with 30 minutes (*p*-value <0.05)

Figure 2. Percent of sperm motility, rapid progression, and viability after ejaculation at laboratory room temperature vs. times (n = 98).

Table 3. CASA systems parameters (VAP, VCL, VSL) in 30, 60, 120, 180, and 240 minutes (n = 98)

Parameter	Time (minutes)	Mean ± SD (%)	*p*-value
VCL (µm/second)	30	76.78±17.97	-
	60	75.57±15.69	0.354
	120	73.26±13.71	0.007*
	180	69.35±12.43	<0.001*
	240	68.13±12.17	<0.001*
VAP (µm/second)	30	47.24±9.55	-
	60	45.83±8.94	0.053
	120	45.07±8.10	0.003*
	180	42.30±7.74	<0.001*
	240	42.26±8.25	<0.001*
VSL (µm/second)	30	35.48±6.89	-
	60	34.82±6.79	0.255
	120	34.85±6.51	0.268
	180	32.97±6.31	<0.001*
	240	33.09±7.09	<0.001*

CASA = computer aided sperm analysis; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity

* Statistical significant if *p*-value <0.05

Table 4. Mean of percent sperm morphology at 30, 60, 120, 180, and 240 minutes after ejaculation (n = 98)

Parameter	Time (minutes)	Mean ± SD (%)	*p*-value
Head defect (%)	30	53.91±7.85	0.03*
	180	55.24±7.81	
Neck defect (%)	30	29.51±6.15	0.29
	180	28.84±6.68	
Tail defect (%)	30	14.73±6.21	0.73
	180	14.56±10.57	

* Statistical significant if *p*-value <0.05

Discussion

The sperm motility is supposed to be one of the most powerful indicators of fertility success in semen analysis. It is also the most useful information to provide appropriate treatments for infertility in male patients. Sperm motility is influenced by both length of time after ejaculation and temperature. Many studies have been conducted to explain these factors that may affect the motility of sperm. Some studies showed the highest sperm motility was achieved when semen was kept and stored at laboratory room temperature[13], while other studies recommended that the optimal temperatures should be equal to the body temperature[15,17]. With regard to sperm viability, some studies found an immediate decline after ejaculation, but the present study summarized that sperm viability could remain intact for two to three hours after ejaculation[18].

In the present study, the authors demonstrated that when semen sample was kept at laboratory room temperature prior to analysis, both, the sperm motility and the percentage of rapid sperms exhibit significant decline within one hour after ejaculation. Furthermore, sperm velocity continued to decrease progressively after one hour. The results in the present study are similar to the experiments carried out by Chomsrimek et al[19]. They also found that there was significant decline in the sperm motility after one hour. Similarly, study conducted by Visconti et al indicated that most sperm had decreased motility, and then became immotile after two hours of incubation[20]. The adenosine triphosphate [ATP], which was the main energy of the sperm, was rapidly consumed. The ATP is formed by glycolysis enzyme and mitochondria in mid piece of sperm. After ejaculation, sperm acquires energy from nutrient molecules such as fructose, sorbitol, mannose, glucose, pyruvate, lactate, and hydroxybutyrate found in seminal fluid. Then the ATP is produced. It is then used in sperm motility and function[20]. Therefore, the sperm become immotile after being out of energy. Interestingly, the authors' present data confirmed the similar changes of the direction of the motility and the vitality.

The one important mechanism to explain the times with semen quality is the reactive oxidative stress [ROS]. The components of seminal fluid released from seminal vesicle and prostate gland consist of estrogen, testosterone, prostaglandins, signaling agents, and some bacterial contamination. Prostaglandins and bacteria in seminal fluid had been reported that they could take the role of ROS. The ROS in the seminal fluid make the negative effect on the sperm parameters that came from heat and toxins when time past. Therefore, bacterial contamination in seminal fluid may compromise the quality of sperm. It has been reported that there is some minimal bacterial contamination in asymptomatic male. For example Eggert-Kruse et al reported that about 71% of semen samples from male test subjects were tested positive for anaerobic microorganisms and potentially pathogenic species although the test subjects exhibited no symptoms of urogenital tract infection[21]. Therefore, many types of gram-positive and gram-negative bacteria were frequently found in semen samples. However, only two species of bacteria (*Escherichia coli* and *Morganella morganii*) have been proven to give a negative effect on sperm motility and viability[22,23]. There are two main mechanisms from the bacteria species, direct pathway and indirect pathway. The direct pathway includes cytotoxic activities that involve bacterial toxin and direct contact between sperm and bacteria. Then, this pathway eventually leads to alteration of mitochondrial activities, DNA degradation, and membrane degradation of sperms[24]. Moreover, the indirect

pathway includes higher level of ROS produced by polymophonuclear neutrophils [PMN] when bacteria contamination was found in semen sample. ROS such as hydrogen peroxide and hydroxyl radical have also been found to be produced directly by bacteria species such as Ureaplasma urealyticum. The previous evidence has shown that presence of defective spermatozoa and dead spermatozoa in seminal fluid leads to elevated activities of ROS. It has been shown that the PMN count of less than 1 million/ml is not necessarily an acceptable indication of normal sperm viability and motility[25-27]. Overall, in the indirect pathway, ROS diminishes intracellular ATP, affecting the process of axonemal protein phosphorylation and impacting the energy level needed for sperm mobilization[28]. However, the explanation of reducing of mitochondrial activity seems to be served as the common target of both the direct and indirect pathways.

Limitation of the present study was some error from minor technical collection of sample such as temperature controlled microscope, basic microscope settings for both bright field and positive phase contrast optics, heating of microscope slides, pipette tips, and any other consumable equipment. The main objectives evaluated the time effect on motility, velocity, and viability of sperm. Other limitations, in morphology of sperm was also explored but the researcher could not correlate to motility parameters of sperm due to limitation on the observational study type. The authors tried to minimize all factors and covariate factors that caused potential bias and confounding such as collection, semen transportation, reviewed techniques, assigned laboratory room temperature, use of the same liquefaction time before sperm analysis, using standardized slides for concentration/motility measurement, and preparing male partners of the infertile couples about pre-period before semen analysis by strictly collecting sample in same abstinence period between two and seven days before collection.

Conclusion

Based on these experiments using the CASA systems, the nature of subjects did not differ from the problem of infertility in the general population. The study showed the sperm velocity and viability begin to be affected after 120 minutes after semen ejaculation, whereas the sperm motility starts to decline after only 60 minutes. Practitioners may well be encouraged to prepare semen samples at laboratory room temperature (25°C to 26°C) in the future. However, for effective and accurate semen analyzes, semen samples should be tested within one hour after ejaculation if the semen sample was stored at laboratory room temperature.

What is already known on this topic?

Both of morphology and motility of sperm are significant factors in predicting fertilization rate and pregnancy rate. Currently, the sperm motility is the most important parameter in evaluating the fertilization ability. WHO guideline stated that semen sample be handled at temperature range between 20°C to 40°C while the most current guideline recommends 37°C. CASA was designed to provide an accurate diagnosis, with precise and significant information of sperm parameters, analyzing the sperm motion parameters at 20°C to 25°C.

What this study adds?

Because the recommendation of storage temperature is controversial, and it is difficult to store the semen sample at 37°C to mimic as body temperature, we studied the effect of time ranges after ejaculation on the motility and viability of sperm at laboratory room temperature of 25°C to 26°C. The time after sperm ejaculation have influenced motility and viability of the sperm. We created a recommendation protocol to get the optimized time to send sample for semen analysis.

Potential conflicts of interest

None.

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ผลของเวลาหลังการหลั่งน้ำอสุจิต่อการเคลื่อนไหวและการมีชีวิตของอสุจิที่อุณหภูมิห้อง ในการตรวจวิเคราะห์น้ำอสุจิ

สิริสุข อุ่ยตระกูล, มัธชุพร สุขประเสริฐ, ฉัตรชัย ตรีธรรมพินิจ, วิชาญ โชคธนะศิริ, ศักดา อาจองค์ วัลลิภากร, ชลธิชา สถิระพจน์

วัตถุประสงค์: เพื่อศึกษาถึงผลกระทบที่มีผลต่อการเคลื่อนไหวและการมีชีวิตของอสุจิ เปรียบเทียบเมื่อเวลาผ่านไป 30, 60, 120, 180 และ 240 นาที หลังการหลั่ง ที่อุณหภูมิห้อง (25-26 องศาเซลเซียส)

วัสดุและวิธีการ: ดำเนินการตรวจวิเคราะห์น้ำอสุจิทั้งหมด 98 ตัวอย่าง ที่อุณหภูมิห้องปฏิบัติการ โดยวิเคราะห์การเคลื่อนไหวของอสุจิด้วยเครื่องคอมพิวเตอร์ (computer aided sperm analysis, CASA) และการมีชีวิตของอสุจิ โดยวิธีทดสอบ eosin ที่เวลา 30, 60, 120, 180, 240 นาที หลังการหลั่งน้ำเชื้อ และทำตรวจสอบรูปร่างของอสุจิโดยวิธี Diff-Quick rapid test ที่เวลา 30 และ 180 นาที

ผลการศึกษา: ผลการศึกษาน้ำอสุจิ 98 ตัวอย่าง จากผู้ชายทั้งสิ้น 70 คน พบว่าค่าเฉลี่ยของการเคลื่อนไหวของอสุจิที่ระยะเวลา 30, 60, 120, 180 และ 240 นาที มีค่า 64.14±14.40, 59.57±16.44, 57.46±16.50, 54.63±18.18 และ 54.60±18.52 เปอร์เซ็นต์ ตามลำดับ พบค่าลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเวลาผ่านไป 60 นาที และพบค่าการมีชีวิตของอสุจิมีค่าดังนี้ 67.59±13.20, 65.88±12.33, 62.10±11, 58.60±14.08 และ 54.10±10.37 เปอร์เซ็นต์ ตามลำดับ และมีการลดลงอย่างมีนัยสำคัญทางสถิติเมื่อผ่านไป 120 นาที

สรุป: จากผลการศึกษา พบว่าการเคลื่อนไหวของอสุจิ จะลดลงเมื่อเวลาผ่านไปนานกว่า 60 นาที อย่างมีนัยสำคัญทางสถิติ ความเร็วในการเคลื่อนตัวและการมีชีวิตของอสุจิจะลดลงอย่างมีนัยสำคัญเมื่อเวลาผ่านไป ประมาณ 120 นาที ดังนั้นการตรวจน้ำอสุจิสามารถทำได้โดยตั้งทิ้งไว้ที่อุณหภูมิห้อง และตรวจไม่เกิน 1 ชั่วโมง หลังจากการหลั่งน้ำเชื้อ เพื่อป้องกันไม่ให้มีผลกระทบกับการเคลื่อนไหวของอสุจิเมื่อนำน้ำเชื้อมาทดสอบ