Effect of Abstinence Duration on Sperm Sex Ratio

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ABSTRACT

Objective: To evaluate the effect of abstinence duration and semen processing on sperm sex ratio and semen parameters.

Methods: 100 normozoospermic samples from men who had abstinence for 1 - 10 days were processed with combined Percoll gradients with swim-up method. The ratio of X- to Y-bearing spermatozoa was determined using fluorescence in situ hybridization. The ratio of X- to Y-bearing spermatozoa and semen parameters in fresh and prepared samples in correlation with abstinence duration were assessed.

Results: No statistically significant influence of abstinence duration on the mean ratio of X- to Y-bearing spermatozoa was detected in fresh or prepared samples. The mean values of total sperm count and total motile sperm in fresh samples were significantly increased after abstinence of more than 3 days. In the prepared samples, the peaks of total sperm count and total motile sperm were noted in day 4 of abstinence while the percentage of morphologically normal spermatozoa remained constant during 1-10 days of abstinence.

Conclusion: There was no statistically significant effect of abstinence duration on the sperm sex ratio in either fresh or prepared samples. The total motile sperm in fresh samples were significantly increased after 4 days of abstinence. After sperm processing, the peak of the total motile sperm was observed at day 4 of abstinence while the abstinence duration did not affect the percentage of morphologically normal spermatozoa.

Keywords: Abstinence duration, sperm sex ratio, Percoll gradients, swim-up

INTRODUCTION

The objectives of sex selection in humans are to prevent sex-linked genetic disorders and to balance sex composition within the family. Many sperm sex pre-selection methods were studied. Flow cytometric separation is the most effective method,[1] but there were some studies which reported this method might cause mutation in the sperm.[2,3] For other selection methods,[4-9] swim-up, discontinuous albumin gradients, Sephadex filtration, or Percoll gradients technique, the sex ratio is not clinically significantly changed.

The natural method, the timing of intercourse in relation to ovulation has some influence on the sex ratio, but it is not a practical method.[10] For gender selection by setting some period of sexual abstinence before the sexual intercourse, the myth was the proportion of Y-bearing spermatozoa would be increased

after sexual abstinence of more than 3-5 days. However, from the previous studies, sperm DNA fragmentation correlated positively with a long abstinence time[11,12] and sperm that carried more DNA fragmentation had less viability rate;[13] while Y chromosome was sensitive to fragmentation and the ability to repair the DNA was extremely limited[12,14,15] so the proportion of Y-bearing spermatozoa should be decreased after longer abstinence duration. To date, there has been no evidence for the effect of abstinence duration on sperm sex ratio.

Moreover there were conflicting data in the effect of sexual abstinence on sperm parameters. From the study of De Jonge C,[16] semen volume and sperm number increased with duration of abstinence, but there was no effect of abstinence duration on sperm motility, morphology, or viability whereas other studies[17-19] found sperm motility and normal morphology significantly decreased with longer duration of abstinence.

MATERIALS AND METHODS

The study was a prospective observational study, conducted in the Division of Infertility, Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. This study protocol was approved by Siriraj Institutional Review Board (COA No.679/2011). From January 2012 to March 2012, men who attended our division for semen analysis were recruited after providing their informed consent. The semen from healthy men, age ≥ 18 years, duration of abstinence 1 to 10 days and normozoospermia samples according to World Health Organization (2010) criteria[20] were included (semen volume ≥ 1.5 mL and sperm concentration ≥ 15x 10^6 /mL). The subjects who had history of receiving radiotherapy or chemotherapy and who had history of abnormal sex chromosome were excluded from the study.

The primary objective was to evaluate the effect of abstinence duration on the ratio of X- and Y-bearing spermatozoa in semen prepared with combined Percoll gradients with swim-up method (combined method) by using fluorescence in-situ hybridization (FISH). The secondary objectives were to evaluate the effect of abstinence duration on sperm sex ratio and semen parameters in the fresh semen; and to evaluate the effect of semen processing on the sperm sex ratio and semen parameters in correlation with abstinence duration.

Semen analysis and preparation

Semen were obtained by masturbation. After liquefaction, semen were analyzed following WHO (2010) guideline. Sperm concentration was evaluated in a hemocytometer chamber. Sperm motility and morphology were examined by using computer-aided sperm analysis (CASA, Hamilton Thorn IVOS system). Sperm vitality was determined after eosin-nigrosin staining. Some portion of fresh semen was reserved for FISH analysis.

After semen analysis was completed, the Percoll-gradient medium (Percoll™ PLUS, GE Healthcare Bio-Science AB, Sweden) was prepared by layering 1 mL of 40% Percoll solution over 1 mL of 80% Percoll solution in a conical centrifuge tube (BD™ Falcon 15 mL polypropylene conical tube, Fisher Scientific, USA). 1 mL of liquefied semen was placed above the Percoll-gradient medium. The whole solution was centrifuged at 300 g for 20 minutes. The supernatant was removed then the pellet was resuspended with 2 mL of sperm washing media (Quinn's Advantage®, SAGE® In vitro Fertilization, Inc. Trumbull, USA). After centrifugation at 300 g for 10 minutes, the pellet was resuspended and recentrifuged. The supernatant was discarded. Some part of the pellet was aspirated for FISH. Thereafter, 1mL of medium was gently placed on the pellet and the tube was placed in a 5% CO_2, 37°C incubator for 30 minutes. Finally the uppermost portion was aspirated for FISH and evaluation of sperm concentration, motility and morphology with CASA. The recovery rate of total motile sperm was calculated as:

$$\frac{\text{Total motile sperm in swim-up fraction}}{\text{Total motile sperm in fresh semen}} \times 100$$

Fluorescence in-situ hybridization (FISH) procedure

FISH was used to determine the proportion of X- and Y-bearing spermatozoa in fresh semen, semen prepared with Percoll gradients method and semen prepared with combined method. These three types of specimen from each subject were mixed with methanol and acetic acid (3:1) and stored at -20°C until ready for FISH procedure.

The semen were smeared onto glass slides and air dried. The FISH procedure was performed using the method described by Kunathikom S et al.[21] Sperm nuclei were decondensed by incubating the slides in 25 mmol/L dithiothreitol (Sigma) and 0.1% trypsin in 2x saline-sodium citrate buffer (SSC) at 37°C for 15 minutes. The slide was periodically visualized under a phase-contrast microscope (Bx40, Olympus, Tokyo, Japan) until the sperm heads were swollen to twice their normal size. Then the slide was rinsed twice with 2x SSC, dehydrated through a series of cold ethanol (70%, 80% and 100%) for 1 minute each, and allowed to air dry. 1 µL of DNA was added to the slide. The samples were then denatured in a humidified chamber (HYBrite™, Abbott Molecular, Inc. IL, USA) at 73°C for 5 minutes and hybridized at 37°C overnight. Commercial DNA probes (XA X/Y/18, MetaSystems GmbH, Altlussheim, Germany), comprising centromeric probes for chromosomes X, Y, and 18, were employed. Post-hybridization washing was performed with following steps: the slides were washed in 0.3% Nonidet P-40 (NP-40, Vysis)/0.4x SSC at 73°C for 2 min, then in 0.1% NP-40/2x SSC at room temperature for 1 min, and allowed to air dry at room temperature. The slides were then counterstained with 125 ng/mL DAPI II solution (Vysis). Finally, the slides were covered with coverslips and sealed with nail polish. The slides were stored at -20°C until ready for evaluation.

The slides were evaluated by two investigators under a fluorescence microscope (Carl Zeiss Metasystem with Isis FISH Imaging System) with 4 single bandpass filters at a magnification of x400. After image capturing, 200 sperm nuclei in each slide were counted, the mean value was considered. If the interobserver variation was more than 10%, repeated evaluations were performed. Sperm nucleus contained one signal of chromosome 18 (aqua) and one of X (green) or Y (orange) chromosome was classified as normal X and Y sperm respectively (Fig 1). Sperm nucleus without any signal was classified as hybridization failure. Sperm which contained only one signal or contained both X and Y signals were not evaluated. The numbers of X- and Y-bearing spermatozoa were calculated.

Fig 1. Photograph of spermatozoa after triple-probe FISH analysis, chromosome 18 (aqua or blue signal), chromosome X (green signal), chromosome Y (orange signal)
A. Normal haploid X-bearing spermatozoa
B. Normal haploid Y-bearing spermatozoa

Statistical method

Values were expressed as mean±SD. ANOVA test was used to determine the differences in the ratio of X- to Y-bearing spermatozoa between abstinence durations, the differences in the ratio of X- to Y-bearing spermatozoa between semen preparation methods and the difference in the semen parameters between abstinence durations. Paired Student's t-test was used to evaluate the effect of semen
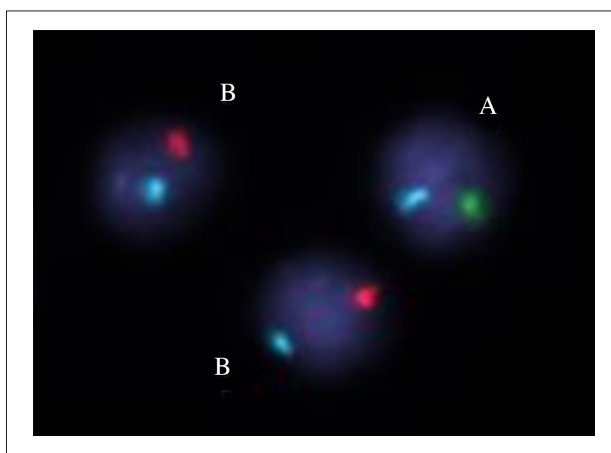
processing by comparing the sperm parameters between fresh semen and semen after prepared with the combined method. Data were tested for normality of distribution.

All statistical analyses were performed using Statistical Programs for Social Sciences (SPSS Inc. version 14.0, Chicago, IL). A p-value <0.05 was considered statistically significant.

Sample size calculation was based on the ratio of X- to Y-bearing spermatozoa in semen which was prepared with the combined method which was 50:50. With a type I error of 0.05 and allowable error of 0.1, at least 97 normozoospermic samples were needed for this study.

RESULTS

During the 3 months of the enrollment period, 185 participants were recruited into the study. 85 participants were excluded due to abnormal semen analysis. Therefore, there were 100 participants who completed the study, 10 cases in each abstinence duration.

The mean age of the participants was 35.34±5.02 years. The hybridization efficiency was 98.50±1.37% in fresh semen, 98.93±0.94%

in semen after prepared with Percoll gradients, and 99.22±0.74% in semen after prepared with combined method.

Abstinence duration and sperm sex ratio

Overall, the mean ratio of X- to Y-bearing spermatozoa were 0.98±0.13, 0.95±0.16, and 0.95±0.12 in fresh semen, in 80% Percoll fraction, and in swim-up fraction, respectively. There was no significant difference from 1:1 ratio in all three groups.

No statistically significant influence of abstinence duration on the mean ratio of X- to Y-bearing spermatozoa was detected in the fresh semen, semen after prepared with Percoll gradients or after prepared with combined method. (Table 1)

Following sperm processing, there was no statistically significant difference in sperm sex ratios between the processed samples and the fresh samples in the same abstinence duration. (Table 1)

Abstinence duration and semen parameters

The mean values of semen parameters in the fresh samples and semen after prepared with combined method have been shown in Tables 2-3.

TABLE 1. The ratio of X- to Y- bearing spermatozoa in fresh semen, in 80% Percoll fraction, and in swim-up fraction in relation to abstinence duration.

Abstinence duration (days)	X:Y ratio			
	Fresh semen	80% Percoll fraction	Swim-up fraction	P value*
1	0.99 ± 0.10	0.98 ± 0.12	0.97 ± 0.10	0.97
2	0.98 ± 0.21	0.93 ± 0.15	0.93 ± 0.14	0.80
3	1.07 ± 0.17	0.95 ± 0.19	0.94 ± 0.11	0.19
4	0.97 ± 0.14	0.84 ± 0.15	0.94 ± 0.05	0.08
5	1.03 ± 0.11	0.99 ± 0.12	1.04 ± 0.13	0.52
6	0.91 ± 0.07	0.94 ± 0.15	0.88 ± 0.10	0.50
7	0.92 ± 0.09	0.93 ± 0.16	0.95 ± 0.12	0.86
8	0.98 ± 0.07	0.90 ± 0.16	0.98 ± 0.18	0.27
9	1.00 ± 0.12	0.93 ± 0.18	0.94 ± 0.16	0.24
10	0.99 ± 0.07	1.06 ± 0.20	0.97 ± 0.08	0.23
P value**	P** 0.19	P** 0.21	P **0.30	

*compared between preparation techniques
**compared between abstinence durations

Semen volume

In fresh semen, the means of semen volume from the abstinence period of 4 days (3.07±1.24 mL) and 7 days (3.14±1.10 mL) were significantly higher than 1-3 days of abstinence.

Sperm concentration

Among fresh samples, there was a significant increase in the mean sperm concentration on day 7 of abstinence (68.00±32.85 x10^6/mL), compared with 2 days of abstinence (41.30±23.83 x10^6/mL). However, no further increase in the mean sperm concentration after 7 days of abstinence was observed.

The prepared samples showed a significant decrease in the mean sperm concentration (32.89±29.26 x10^6/mL), compared with the fresh samples (58.66±30.44 x10^6/mL). The peak of mean sperm concentration in prepared samples was observed on day 4 of abstinence (54.20±70.37 x10^6/mL). This value was also significantly higher than the mean values of day 1 (23.40±19.40 x10^6/mL), day 2 (22.30±11.40 x10^6/mL) and day 9 of abstinence (19.50±8.48 x10^6/mL).

Total sperm count

The peaks of the mean total sperm count per ejaculate in the fresh samples were noted on day 4 (220.43±180.74 x10^6) and day 7 (203.51±97.95 x10^6) of abstinence which were significantly higher than 1-3 days of abstinence.

There was a significant difference in the mean of total sperm count per ejaculate between fresh sample (149.46±107.39 x10^6) and prepared samples (16.44±14.63x10^6).

Among prepared samples, the peak mean total sperm count was noted on day 4 of abstinence (27.10±35.19 x10^6). This value was also significantly higher than the mean values of day 1 (11.70±9.70 x10^6), day 2 (11.15±5.70 x10^6) and day 9 of abstinence (9.75±4.24 x10^6).

Sperm motility

In fresh samples, the peak mean sperm motility was obtained after abstinence for 4

TABLE 2. Comparison of the mean values of semen parameters in fresh semen in relation to abstinence duration.

Abstinence duration (days)	Volume (mL)[a]	Concentration (x10^6/mL)[b]	Total sperm count (x10^6)[c]	Motility (%)[d]	Total motile sperm (x10^6)[e]	Normal morphology (%)	Viability (%)[f]
			Semen parameters				
1	2.08 ± 0.61	47.69 ± 30.59	96.31 ± 57.10	61.60 ± 11.87	59.45 ± 39.05	18.10 ± 4.82	75.50 ± 8.15
2	2.28 ± 0.70	41.30 ± 23.83	97.47 ± 74.51	53.10 ± 12.91	53.48 ± 42.03	14.70 ± 2.54	71.80 ± 7.50
3	2.08 ± 0.46	50.10 ± 22.85	106.40 ± 62.01	51.10 ± 7.14	56.79 ± 37.06	15.30 ± 1.70	75.30 ± 4.92
4	3.07 ± 1.24	63.90 ± 28.57	220.43 ± 180.74	64.20 ± 14.12	151.18 ± 133.91	15.80 ± 6.12	76.50 ± 7.55
5	2.65 ± 1.18	61.20 ± 31.06	156.45 ± 97.49	56.40 ± 8.72	87.04 ± 50.56	17.70 ± 5.27	77.90 ± 5.90
6	2.56 ± 0.91	43.90 ± 17.97	112.15 ± 60.80	45.10 ± 9.23	51.19 ± 30.08	18.00 ± 6.09	70.40 ± 7.86
7	3.14 ± 1.10	68.00 ± 32.85	203.51 ± 97.95	51.80 ± 6.89	106.05 ± 60.87	19.70 ± 8.63	69.20 ± 9.89
8	2.20 ± 0.77	70.28 ± 36.10	156.99 ± 127.95	50.80 ± 9.35	86.66 ± 88.34	15.20 ± 4.83	75.60 ± 4.50
9	2.68 ± 1.41	70.70 ± 34.73	185.44 ± 120.93	55.10 ± 13.30	102.21 ± 65.33	15.60 ± 7.59	74.50 ± 13.31
10	2.33 ± 0.61	69.50 ± 33.27	159.44 ± 86.48	51.40 ± 10.81	82.30 ± 47.56	15.40 ± 5.87	71.50 ± 8.37

[a] P<0.05 from day 1-3 and day 8 to day 4 and day7, [b] P<0.05 from day 2 to day 7-10, [c] P<0.05 from day 1-3 to day 4 and day 7, [d] P<0.05 from day 1 and day 4 to day 6-8 and day 10, from day 2-3 to day 10, [e] P<0.05 from day 1-3, day 5, 6, 8, 10 to day 4, [f] P<0.05 from day 4-5 to day 7, from day 5 to day 6

TABLE 3. Comparison of the mean values of semen parameters in semen prepared with combined method in relation to abstinence duration.

Abstinence duration (days)	Semen parameters				
	Concentration $(x10^6/mL)^a$	Total sperm count $(x10^6)^b$	Motility $(\%)^c$	Total motile sperm $(x10^6)^d$	Normal morphology (%)
1	23.40 ± 19.40	11.70 ±9.70	92.40 ± 3.34	10.70 ± 8.82	22.40 ± 6.60
2	22.30 ±11.40	11.15 ± 5.70	92.30 ± 5.85	10.47 ± 5.69	20.50 ± 9.40
3	36.90 ± 19.26	18.45 ± 9.63	88.90 ± 4.86	16.58 ± 9.14	22.30 ± 5.62
4	54.20 ± 70.37	27.10 ± 35.19	86.40 ± 6.24	22.88 ± 27.93	24.60 ± 7.29
5	37.20 ± 21.16	18.60 ± 10.58	91.70 ± 3.95	16.83 ± 9.33	24.70 ± 5.42
6	28.86 ± 25.05	14.43 ± 12.52	91.80 ± 2.49	13.07 ± 11.11	24.20 ± 8.04
7	41.90 ± 26.63	20.95 ± 13.31	89.10 ± 7.32	18.35 ± 11.59	26.50 ± 8.33
8	32.70 ± 19.01	16.35 ± 9.51	91.60 ± 2.55	14.96 ± 8.68	24.30 ± 10.27
9	19.50 ± 8.48	9.75 ± 4.24	91.10 ± 9.43	9.03 ± 4.41	19.80 ± 9.20
10	31.91 ± 17.16	15.96 ± 8.58	90.30 ± 5.60	14.33 ± 7.81	22.20 ±6.09

[a] P<0.05 from day 1, 2, and 9, to day 4, [b] P<0.05 from day 1,2, and 9 to day 4, [c] P<0.05 from day 1, 2, 5, 6, and 8 to day 4, [d] P<0.05 from day 1, 2, and 9 to day 4

days (64.20±14.12%). There was a significant decrease of this value in the group of 1 to 3 days ofabstinence, compared with 4 days of abstinence. The mean sperm motility from 1 day of abstinence was significantly higher than abstinence interval of 6-10 days.

The mean sperm motility increased from 54.06±11.61% in fresh to 90.56±6.61% after prepared with the combined method whereas, the abstinence duration did not have any effect on the mean sperm motility in prepared samples.

Total motile sperm

The mean total motile sperm per ejaculate in the fresh samples was statistically significantly higher after abstinence for 4 days $(151.18±133.91 \times 10^6)$ in comparison with the samples collected after other abstinence durations. The low mean total motile sperm was observed on days 1-3 of abstinence.

After sperm preparation with combined method, the recovery rate of motile sperm was 22.85±15.89%. The mean total motile sperm per ejaculate peaked at day 4 of abstinence (22.88±27.93), like the mean of total sperm count.

Sperm morphology

Among the fresh samples, there was no significant difference in the mean percentage of morphologically normal spermatozoa between abstinence durations.

A significant improvement in the mean percentage of morphologically normal spermatozoa was observed after sperm preparation (16.55±5.67% in fresh samples and 23.15±7.69% in prepared samples). However, no statistically significant influence of abstinence duration on this parameter was demonstrated in prepared samples.

Sperm viability

The mean sperm viability among the fresh samples was significantly decreased at day 6 (70.40±7.86%) and day 7 of abstinence (69.20±9.89%), compared with day 4 of abstinence (76.50±7.55%).

DISCUSSION

Abstinence duration and sperm sex ratio

The inclusion criteria of normozoospermia according to WHO criteria (2010) can be applied for the men who receive in vitro fertil-

ization for prevention of sex-linked disorders in the offspring and who wish to balance sex ratio in their family with the natural method. Moreover this criterion can exclude the men who had sex chromosome abnormalities because the patients with these disorders often had oligozoospermia.

Fluorescence in-situ hybridization is the accurate method for determining the ratio of X- to Y-bearing spermatozoa because it uses specific DNA probes for each chromosome.[22] In the present study, chromosome 18 was used as internal control which helped to differentiate nullisomy from hybridization failure.[23]

Because of the inability to have homologous recombination repair,[14] Y chromosome favors accumulated transposable elements which makes it unstable.[24] Moreover, spermatozoa are deficient in both antioxidant and DNA-repair systems so oxidative stress can cause additional DNA fragmentation in Y-bearing spermatozoa. From the study of Richthoff J et al,[11] DNA fragmentation index correlated positively with abstinence time (r=0.17). Furthermore, Ozmen B et al,[13] had demonstrated the negative correlation between increased DNA damage and viability of sperm. Therefore, the Y-bearing spermatozoa might be susceptible to decreasing viability after a long abstinence period.

This is the first study to evaluate the effects of abstinence period on sperm sex ratio. Based on our hypothesis, the ratio of X- to Y-bearing viable spermatozoa from long abstinence period should be higher than the samples from short period of abstinence.

From the present study, the ratio of X- to Y-bearing spermatozoa in fresh semen was nearly 1:1 among different abstinence periods because the fresh specimen composed of both live and dead spermatozoa, so the ratio between X- and Y-bearing spermatozoa was 1:1 according to spermatogenic meiotic division principle. Sperm preparation methods were performed in order to select only the viable spermatozoa. We used the combined Percoll gradients and swim-up method to process the semen because

this technique can select highly motile, morphologically normal spermatozoa with lower percentage of sperm DNA fragmentation in comparison with density gradient centrifugation alone.[25,26]

The principle of sperm preparation with Percoll gradients is based on a combination of sperm motility and the retention of spermatozoa at the phase border.[27] This technique can select the motile, viable spermatozoa with good chromatin integrity.[28] On the basis of the difference in DNA content between X and Y chromosomes, the X chromosome is 160 Mb long, while the Y chromosome is only 60 Mb,[14] but there is no difference in the size between X- and Y-bearing spermatozoa. Therefore, the density of X-bearing spermatozoa should higher than Y-bearing spermatozoa. Additionally, X-bearing spermatozoa were more likely to have less DNA damage than Y-bearing spermatozoa. Thus Percoll gradients method might be able to separate the two types of sperm.

The result of our study demonstrated that the ratio of X- to Y-bearing spermatozoa was not significantly different between fresh samples and the semen after prepared with Percoll gradients method. This finding was compatible with the previous studies.[7-9] The two possible explanations for the indifference were, firstly, the differences in DNA content and DNA damage between X- and Y-bearing spermatozoa were too small to show the difference in the density of spermatozoa, and secondly, spermatozoa with good motility did not represent spermatozoa with good DNA integrity.[28]

After the semen samples were further processed with swim-up method, the mean ratio of X- to Y-bearing spermatozoa was still not different from 1:1. This finding was similar to the previous studies.[4-6] The results of the previous studies showed no significant change in the distribution of X- or Y-bearing spermatozoa following swim-up technique from the unprocessed controls. This may be explained by the indifference in swim-up velocity between X- and Y-bearing spermatozoa.

There may be other factors that can interfere with the efficacy of sperm preparation techniques such as changing of sperm density after sperm maturation,[29] discrepancy in sperm morphology, and random sperm motility.[30] Hence it might not be possible to separate the X- and Y-bearing spermatozoa with Percoll gradients or swim-up method.

Among the prepared samples, abstinence duration did not effect on the sperm sex ratio. This result might be because the normozoospermic samples contained few DNA fragmentation, so the viability between X- and Y-bearing spermatozoa from different abstinence periods may not be significantly different.

Abstinence duration and semen parameters

The effect of abstinence duration on semen parameters was also evaluated. In fresh semen, the mean value of semen volume, sperm concentration, and total sperm count were significantly increased after abstinence of more than 3 days. Because of the increase in mean total sperm count after 3 days of abstinence, the mean total motile sperm also significantly increased in the same trend despite the mean percentage of sperm motility was significantly decreased after that period. There was a statistically significant decrease in the mean sperm viability when the abstinence period was more than 5 days, while the abstinence duration did not affect the mean percentage of morphologically normal spermatozoa.

The results regarding the semen volume, sperm concentration, total sperm count, sperm motility, total motile sperm, and sperm morphology were nearly compatible with data reported by many previous studies.[16,19,31] From the retrospective study of Levitas E, et al,[19] there was a significant increase in semen volume after day 5 of abstinence, and the mean sperm concentration significantly increased starting on day 3 of abstinence and continued through day 6; a significant increase in mean total sperm count was observed between day 1 and day 7 of abstinence; there was also a significantly increase in the mean total sperm count on every additional day of abstinence from day 3 to day 6 of abstinence; and there was a significant improvement in mean percentage of sperm motility after 1 day of abstinence, but a decrease in percentage of sperm motility was observed after day 8-10 of abstinence with a further significant decrease after 11-14 days; mean total motile sperm gradually increased from day 0-7 and a steady level of normal sperm morphology was observed on the succeeding abstinence days. In contrast, de Jonge C, et al,[16] and Carlsen E, et al,[32] did not find any influence of abstinence duration on sperm motility, morphology, or viability. These studies had evaluated the effect of abstinence duration on within-subject semen parameters whereas our study and the study from Levitas E, et al, analyzed inter-subject semen parameters.

Due to the prolonged stasis of spermatozoa in the epididymis, the spermatozoa were likely to be exposed to reactive oxygen species (ROS) from leukocytes and dying spermatozoa. This ROS attacks the integrity of DNA[33] which can deteriorate sperm motility and viability.[13, 34-37]

From the evidence mentioned above, it could be used to explain why the mean values of sperm concentration and total sperm count in prepared samples were decreased after long abstinence duration.

Not surprisingly, the improvement in the mean percentage of motile spermatozoa and mean percentage of morphologically normal spermataozoa were noted after sperm processing with the combined method. There is no significant difference in these two parameters among abstinence durations.

After sperm preparation with the combined method, the recovery rate of total motile spermatozoa was poor (22.85±15.89%) in comparison with the preparation method of Percoll gradients alone or swim-up method alone. From the study of Chen S, et al,[38] their recovery rates of total motile spermatozoa were 55.9±4.4% after Percoll gradients and 26.7±3.5% after swim-up method. The decreases in the mean percentage of sperm motility and the mean of total motile sperm after day 4 of abstinence

could be explained by the significant decrease of sperm viability after days 6-7 of abstinence. Although the mean total motile spermatozoa in prepared semen from our study were only $14.71\pm12.24 \times10^{6}$, the amount of spermatozoa was enough for both in vitro fertilization and intrauterine insemination so normozoospermic men can be abstinent for up to 10 days before sperm preparation for assisted reproduction. However, in oligozoospermic men, this preparation method might not yield adequate sperm for intrauterine insemination.

Our study had some limitations. The numbers of sperm scored in each slide were only 200 nuclei, and higher numbers may give more accuracy. The semen samples in this study were obtained from different participants, so the results could not represent within-subject variation on sperm sex ratio and sperm paramters.

CONCLUSION

There was no statistically significant effect of abstinence duration on the ratio of X- to Y-bearing spermatozoa in either fresh normozoospermic samples or semen after prepared with combined Percoll gradients with swim-up method. Semen volume, sperm concentration, total sperm count, and total motile sperm were significantly increased after 4 days of abstinence. The process of combined method could improve the percentages of sperm motility and morphologically normal spermatozoa, but the recovery rate of total motile sperm was low. The peak of total motile sperm was observed at day 4 of abstinence. The percentage of morphologically normal spermatozoa in both fresh and prepared samples remained constant during 1-10 days of abstinence.

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