Semen quality of 1,559 young men from four cities in Japan

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Title:

Semen quality of 1,559 young men from four cities in Japan

Study design:

Cross sectional population-based study

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ARTICLE SUMMARY

Article focus:

- There has only been one Japanese study reporting semen quality of presumably normal young men indicating good semen quality compared with men from Europe.
- To establish the frequency of impaired semen quality among normal young Japanese men.

Key messages:

- Semen quality of the young men was significantly poorer than that of partners of pregnant women, and 32% may have reduced fertility chances.
- Semen quality was highly variable.
- These results will serve as a reference for future studies on time trends in semen quality in Japan and for comparison with future studies of university students in other countries.

Strengths and limitations:

- Large-scale prospective study of semen quality among Japanese young men.
- Standardised inclusion criteria and investigation procedures.
- Lack of influence of ethnicity and genetic background on semen quality.
- Relatively low participation rate limits the possibility to generalize the results for the whole population, which is the common problem for all semen studies

ABSTRACT

Objectives: To provide information of semen quality among normal young Japanese men and indicate the frequency of reduced semen quality.

Design: Cross-sectional, coordinated studies of Japanese young men included from university areas. The men had to be 18 to 24 years, and both the man and his mother had to be born in Japan. Background information was obtained from questionnaires. Standardized and quality controlled semen analyses were performed, reproductive hormones analyzed centrally and results adjusted for confounding factors.

Setting: Four study centres in Japan (Kawasaki, Osaka, Kanazawa and Nagasaki).

Participants: 1,559 men, median age 21.1 years, included during 1999-2003.

Outcome measures: Semen volume, sperm concentration, total sperm count, sperm motility, sperm morphology and reproductive hormone levels.

Results: Median sperm concentration was 59 (95% confidence interval 52-68) million/mL, and 9.0% and 31.9% had less than 15 and 40 million/mL, respectively. Median percentage of morphologically normal spermatozoa was 9.6 (8.8-10.3) %. Small but statistically significant differences were detected for both semen and reproductive hormone variables between men from the four cities. Overall, the semen values were lower than those of a reference population of 792 fertile Japanese men.

Conclusions: Assuming the investigated men were representative for young Japanese men, a significant proportion of the population had suboptimal semen quality with reduced fertility potential, and as a group they had lower semen quality than fertile men. However, the definitive role – if any - of low semen quality for subfertility and low fertility rates remains to be investigated.

INTRODUCTION

Whether there has been a global decreasing trend in semen quality remains still controversial. In contrast, there is a consensus that regional differences exist.[1-7] Cross-sectional, coordinated studies of young men from the general populations have shown that men from the Western part of the Northern European countries have lower semen quality than men from the Eastern part or men from Southern Spain, [4-8] which is inversely correlated with the testicular cancer incidences.[8 9]

There has only been one Japanese study reporting semen quality of presumably normal young men[10] indicating good semen quality compared with men from Europe. However, the authors concluded that their results might be flawed by a selection bias and lack of ability to account for confounding factors, and they requested well-designed prospective studies to be performed in several regions of Japan.

Here, we present the results of prospectively designed, cross-sectional studies of young university students from four different provinces in Japan. Our objectives were to elucidate if reduced semen quality were frequent among Japanese men unselected for their fertility status, to examine possible regional differences, to provide a reference for future studies on time trends in semen quality, and to compare the results with those obtained in other countries.

METHODS

The investigations took place at four study centres based in departments of urology at university hospitals in Kawasaki, Osaka, Kanazawa and Nagasaki in Japan. The investigation procedures described below were the same as those of the previously published European studies[4 5 7] except for the selection of study populations and assessment of semen volume.

Study populations of young men

University students were informed about the study through posters placed in several conspicuous places on the campuses of the universities connected to the four study centres. Serially numbered leaflets giving detailed information about the study were attached to the posters. A candidate volunteer had to call the study centre, inform the serial number printed on his copy of the leaflet to make an appointment for the investigations approximately one week later, and to receive a package of documents within days by mail. The package included further written information, a questionnaire and the instruction to preferably abstain from ejaculation for at least 48 hours prior to producing the semen sample for the study. Further inclusion criteria were that the man was 18-24 years, and that both he and his mother were born in Japan. Failure to comply with the request for ejaculation abstinence period was not a reason for exclusion, but the abstinence time was recorded according to the information given by the study subjects at the time of the semen sample delivery. On the day of attendance that was set at a certain time in the morning, the man returned the completed questionnaire, underwent a physical examination, provided a semen sample and had a blood sample drawn.

The study in Kawasaki covered two separate periods: May 1999 to April 2000 and April 2002 to May 2003, and the study periods in Osaka, Kanazawa, and Nagasaki were September 2002 to October 2003, July 2002 to June 2003, and July 2002 to July 2003, respectively. In total 9,374 leaflets were taken by the students and 1,559 (16.6%) participated; 14.5% (658/4,534) from Kawasaki, 11.7% (300/2,570) from Osaka, 21.9% (300/1,371) from Kanazawa, and 33.3% (301/899) from Nagasaki.

Questionnaires

The questionnaire included information on age and previous or current diseases, including any known history of fertility. To assure the quality of the information regarding previous

conditions the men were asked to fill in the questionnaire – if possible – in collaboration with their parents.

Physical examination

The participants had their testes size measured by use of a Prader orchidometer (Pharmacia & Upjohn, Copenhagen, Denmark). The presence of varicocele or other scrotal abnormalities and the Tanner stage of pubic hair were evaluated. Body weight and height were self-reported, and body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters.

Semen samples

The ejaculation abstinence period was calculated as the time between the current and previous ejaculation based on self-reported information from the men. Semen samples were collected at the laboratory and kept at 37°C during liquefaction. Semen volume was assessed by aspirating the entire sample into a graduated 5ml syringe (TERUMO, Tokyo). Sperm motility was assessed on 10 µl of well-mixed semen placed on a clean glass slide, covered with a 22x22 mm coverslip, and then examined at a total magnification of 400 times on the heating stage at 37°C of a microscope. The sperm were classified as either motile (WHO motility classes A, B or C) or immotile (class D), in order to record the proportion of motile sperm.[11] The motility assessment was repeated on a second 10 µl aliquot of semen, and the average value of the two samples was calculated. For the assessment of sperm concentration the samples were diluted in a solution of 0.6 mol/L $NaHCO_{33}$ and 0.4% (v/v) formaldehyde in distilled water, subsequently assessed using Bürker-Türk haemocytometers. Only sperm with tails were counted. Smears were prepared for morphological evaluation, Papanicolaou stained and finally assessed according to strict criteria[12] by one examiner (MV) in Finland between 2009 and 2010.

Quality control of sperm concentration assessment

Inter-laboratory variation in assessment of sperm concentration was monitored by an external quality control (QC) programme coordinated by the Department of Growth and Reproduction Copenhagen, Denmark[3 4] during the study period.

Blood samples

A blood sample was drawn from a cubital vein of each participant usually in the morning to reduce the effect of diurnal variation in hormone levels, and the serum was separated by centrifugation after clotting and stored at –20°C. The frozen serum was sent to the Department of Growth and Reproduction, Rigshospitalet, in Copenhagen, Denmark for a centralised hormone analysis. Levels of testosterone, FSH, LH and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). Inhibin-B was measured by a specific two-sided enzyme immunometric assay (Serotec, UK). Intra- and inter-assay coefficients of variations (CV) for measurements of both FSH and LH were 3% and 4.5%, respectively. CVs for both testosterone and SGBG were <8% and <5%, respectively. The intra- and inter-assay CVs for inhibin-B were 15% and 18%. Free testosterone (cFT) was calculated from total testosterone and SHBG using fixed albumin level of 43.8 g/L as described by Vermeulen.[13]

Comparison population: Fertile men (partners of pregnant women)

From January 1999 to February 2002, our group also examined the semen quality of 792 fertile men (partners of pregnant women) (a manuscript describing these have been submitted in parallel). Participation of these men was similar to that of current study population of young men: they answered a questionnaire, delivered a semen sample, and had a physical examination performed. The results of their semen analyses were used for comparison to that of the young men.

Statistical analysis

Standard statistics (mean, median, standard deviation (SD), 5-95 percentiles and frequencies) were used for description (Tables 1-3). Between-group differences for continuous variables giving the basic description of the study population were tested by the non-parametric Kruskal-Wallis test. Between-group differences for categorical variables were tested with the Fisher's exact test.

The main outcome variables were the assessed semen and hormone variables, and the between group differences were tested by multiple linear regression (Tables 2 and 3). Semen volume, sperm concentration and total sperm counts were best normalised by cubic root transformation before analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa were logit-transformed. Percentages of morphologically normal spermatozoa entered the model untransformed. Ejaculation abstinence up to 96 hours had a linear increasing effect and abstinence above 96 hours a slight, but significant non-linear increasing effect on semen volume, sperm concentrations and total sperm counts. Abstinence therefore entered the model as a covariate as linear splines and abstinence-squared for the part above 96 hours. For motility, winter season was associated with lower motility percentages and season was therefore included as a covariate. For all semen variables increasing age tended to be slightly, but negatively associated with semen variables and age was also included in the models. Duration from ejaculation to assessment was additionally evaluated as a confounder for motility, but found to be non-significant, and therefore not included.

Natural logarithmic transformation gave models in which differences between centres and effects of covariates are more easily interpretable. This alternative model approximate closely the model obtained by cubic root transformation and is used when reporting adjusted semen volumes, sperm concentrations and total sperm counts to represent a 21-year-old man having an ejaculation abstinence period of 96 hours. QC results did not show any significant inter-laboratory differences, changes during the study period or difference to the

reference laboratory. Therefore, corrections of data were not needed to make them comparable. The logit-transformed motility data and untransformed morphology percentages were used to give adjusted levels for a 21-years-old man for these variables.

Differences with $p<0.05$ were considered statistically significant. All statistical analyses were done twice: MNM using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and NJ using PASW version 18.

RESULTS

A description of the study population is summarized in Table 1. Few men had caused a pregnancy or experienced infertility problems, in total 4.7%. During the preceding 3 months to participation in the study, 10.4% had used medications which were mainly antibiotics, painkillers, asthma or allergy medicines.

Semen results are shown in Table 2. “Observed” values are based on raw data, and “Adjusted” are the estimates from regression analyses taking covariates into account. Sperm concentrations did not differ between men from the four study centres, whereas the semen volume for men from Kanazawa was higher than in other centres ($p<0.0001$-0.02 in pair-wise comparisons). Consequently, also total sperm counts were higher for these men, but only significantly in the pair-wise comparison with men from Kawasaki ($p<0.02$). The percentages of motile spermatozoa differed significantly, because men from Nagasaki had higher frequencies of motile spermatozoa than men from other centres (adjusted medians 64-75%). The percentage of morphologically normal spermatozoa for men from Nagasaki was higher than that from Osaka ($p<0.0001$ in pair-wise comparison).

Cumulated, 2.2% of men had a sperm concentration below 5 million/mL, 4.9% below 10 million/mL, 9.0% below 15 million/mL and 31.9% below 40 million/mL. For morphology 5.7% of men had normal spermatozoa below 5%. Figure 1 summarizes the distribution of the sperm concentration, total sperm counts, percentages and total numbers of morphologically normal spermatozoa.

Figure 1 summarises the semen results of the young men in comparison to that of the 792 fertile men. The semen variables differed between these groups, with fertile men having higher semen volume ($p<0.002$), sperm concentration ($p<0.0001$), total sperm count ($p<0.0001$), total number of morphologically normal spermatozoa ($p<0.0001$) than young men, while the percentage of normal spermatozoa did not differ between the groups ($p=0.05$).

The semen variables of men whose mothers had smoked during pregnancy did not significantly differ from non-exposed men, but the number of smoking mother was very small. The men's own smoking or drinking habits (not shown in Table), previous experience of cryptorchidism, testicular torsion, orchitis, sexually transmitted diseases (chlamydia, gonnorhoea or epididymitis) or previous fertility experience did not affect their semen variables.

Reproductive hormone levels differed between the groups of young men (Table 3). Men from Kawasaki and Osaka had slightly higher FSH ($p<0.0001$-0.049) and inhibin-B ($p<0.003$-0.01) levels than men from Kanazawa and Nagasaki. A similar pattern was seen for total testosterone (all $p<0.0001$), SHBG (all $p<0.0001$) and cFT (all $p<0.0001$).

For the entire group of men the median (5-95 percentiles) sizes of the left and right testes were both 22 ml (14-28 ml). The average testis size (mean of left and right) differed significantly between the study groups, ranging from 19 to 23 ml in medians (Table 1). Overall 98.8% of study subjects had a pubic hair distribution of Tanner stage 4 or higher: Kawasaki 98.2%, Osaka 98.0%, Kanazawa 100% and Nagasaki 99.7%.

During the physical examination 27.1% of men was diagnosed with a varicocele; 14.3% stage 1, 9.1% stage 2, and 3.7% stage 3. Varicocele was on the left side in 26.5% and on the right in 3.5% of men. In 9 men (0.6 % of the entire study population) the right varicocele was not concomitant with a left one. The presence of a varicocele was non-significantly associated with an 8% (95% confidence interval –20%;+6%) reduction in sperm concentration and 11% decline (-23%;+4%) in total sperm count. No tendencies

could be detected for sperm motility or morphology. Inhibin-B and total testosterone tended to be lower in men having a varicocele, however, non-significantly ($p>0.05$).

DISCUSSION

Sperm concentration, total sperm count, percentage of morphologically normal spermatozoa and percentage of motile spermatozoa varied tremendously between individual participants. However, only small but statistically significant differences were detected for both semen and reproductive hormone levels between young men from the four provinces in Japan. Thus, from a biological point of view these groups of men can be regarded as similar. The most important finding is that semen quality of the young men was significantly poorer than that of partners of pregnant women.

To our knowledge, this is the first large-scale prospectively designed study to explore potential differences in testicular function parameters in Japanese men that were not selected by any fertility status. The men were enrolled by the same type of advertisement. They were all university students. We tested for effects of various covariates and accounted for these when necessary. Thus, the comparison between the four groups is valid, and the results may serve as a reference for future studies on time trends in semen quality.

In Japan, there is not a compulsory medical examination of young men as in the Northern-European countries,[4 7] which makes it difficult to recruit men that are representative for the general population. Itoh *et al* [10] detected a median sperm concentration of 81 million/mL in 207 young men (18-22 years old) examined in 1998 in Sapporo but also concluded that selection bias in the recruitment and variable ejaculation abstinence might have affected the results. In the design of our study, we therefore decided to restrict the invitation to university students to get a well-characterized study population. Men who have experienced fertility problems may be more likely to volunteer for semen studies than men without any problems.[14] We restricted the age of participants to 18-24 years assuming that the majority in such a group would not yet have any direct knowledge about their own fertility

chances, since the average Japanese man fathers his first child at the age of 31.8 years.[15] Less than 1% of the participants had experienced fertility problems, and such problems are therefore unlikely to severely bias the obtained results. Poorly educated men may be more likely to refuse participation in studies that require delivery of semen samples.[16] However, higher educational level has been positively associated with semen quality.[16 17] Thus, the selection of university students may bias the results towards a higher level.

There were small differences in inhibin-B levels that were lower in men from Kanazawa and Nagasaki than in men from Kawasaki and Osaka. Similar trends were detected for total testosterone, SHBG, and cFT. The slightly higher inhibin-B levels in men from Kawasaki and Osaka could theoretically indicate a higher spermatogenic activity in these, which however does not fit with the sperm count findings. Also, we hesitate to draw such a conclusion since the detected inhibin-B concentrations at this level do not correlate strongly with sperm counts.[18] We have no explanation for the higher total testosterone in men from Kawasaki and Osaka compared to other centres. It is important to keep in mind that the adjusted hormone levels in Table 3 were adjusted for the effect of BMI, which thus does not explain the findings.

Diagnostic accuracy of varicocele is very dependent on the clinical experience of the investigator. The high frequency of varicocele may reflect this, since the majority of the cases were grade 1. We saw no significant negative effect on semen quality, which however, is likely due to the low grade in most varicoceles.

In the European studies semen volume was assessed by weighing which now is the "gold standard", because aspiration – as used in our study – underestimates the volume by approximately 0.4 mL.[19 20] Thus, the true median semen volume might have been 3.4 mL, which is closer to the volumes reported from Finland, Denmark, Germany, and Spain,[3 7 8] and thus the adjusted median total sperm count in our study would have been 200 million rather than 177. Inter-observer variation may explain the higher semen volume detected in men from Kanazawa compared to other centres, because volume measurements were not under external quality control.

Three Chinese studies have described semen qualities in apparently normal men. Healthy men, 20-60 years of age, had a median sperm concentration and total sperm count of approximately 65 million/mL and 154 million, respectively[21]. The youngest age group, 20-25 years old, comprised only 6.1% of the study population, and overall 83% had previously fathered a child. Another study investigated 20-40 years old men but excluded those with known andrological diseases and reported a median sperm concentration and total sperm count of 78 million/mL and 168 million.[22] Junqing *et al*[23] detected a mean sperm concentration and total sperm count of 55 million/mL and 124 million, respectively, in 22-30 years old men that underwent a premarital physical examination. Thus, the latter may be more comparable to ours, since the men presumably had little knowledge of their fertility potential. These Chinese reference values are clearly lower than the current Japanese figures, suggesting that there are regional differences in semen quality among Asian men. Interestingly, Chinese men who had at least a college education had lower sperm counts than men with a lower educational status.[23] If this were true also for Japanese men, the difference in semen quality between Japanese and Chinese men would be even greater than what is evident from the present results.

Other studies have shown an adverse effect of maternal smoking during pregnancy on the son's semen quality.[24-26] However, we did not find such an effect, probably because the number of smoking mothers was very small.

The Japanese men appeared to have higher sperm counts than men from the Northern Europe[4 5 7 27-30] but slightly lower than men from Spain.[8] Thus, Japanese men may be ranked as having better sperm counts than many populations of European young men. Nevertheless, this does not imply that impaired semen quality is not a problem among young Japanese men. Nearly 10% had sperm counts below the current WHO reference levels of 15 million/mL or 39 million[30] rendering them for high risk of reduced fertility in the future. Nearly one third of men had sperm concentration less than 40 million/mL, indicating reduced fecundity.[32-35] We are therefore concerned that a significant proportion of the men may experience problems when they reach the ages when they want to reproduce.[32-35]

Our prospectively designed study showed no overall difference between men from the four investigation sites, and the semen results were similar to those previously reported for Finnish men that until now have been considered to have the best semen quality among young men from the Northern Europe. The study cohorts were, however, not completely comparable. The recently detected adverse trends in semen quality and testis cancer incidences among men from Finland suggest underlying environmental causes.[30] Whether the semen quality of Japanese men has changed over the years cannot be answered by our current study, but a large proportion of investigated men were shown to have sub-optimal sperm counts. These results will serve as a reference for future studies on time trends in semen quality in Japan and for comparison with future studies of university students in other countries.

Table 1: Physical appearance and self-reported information of young men from four cities in Japan.

	Entire study population (n=1559)		Kawasaki (n=658)		Osaka (n=300)		Kanazawa (n=300)		Nagasaki (n=301)		
	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	p-values
Height (cms)	172 (6)	172 (163-181)	172 (6)	172 (163-182)	172 (6)	172 (163-181)	173 (6)	173 (163-181)	172 (5)	171 (163-180)	0.01 [A]
Weight (kgs)	64 (9)	63 (52-79)	64 (11)	63 (52-80)	63 (8)	63 (52-77)	64 (8)	63 (52-80)	63 (9)	62 (52-82)	0.4 [A]
BMI (kg/m2)	21.4 (2.7)	21.0 (18.2-26.0)	21.5 (3.0)	21.0 (18.2-26.0)	21.2 (2.2)	20.9 (18.2-25.4)	21.4 (2.4)	21.1 (18.4-25.8)	21.6 (2.6)	21.3 (18.0-26.2)	0.2 [A]
Mean of left and right size (ml) [a]	21 (4)	22 (14-28)	22 (4)	22 (15-28)	22 (5)	23 (14-29)	21 (4)	21 (14-29)	20 (4)	19 (13-26)	<0.0001 [A]
Age (years) [b]	21.3 (1.6)	21.1 (18.9-24.1)	20.8 (1.4)	20.7 (18.7-23.4)	21.7 (1.6)	21.6 (19.3-24.4)	21.8 (1.6)	21.9 (18.9-24.2)	21.3 (1.7)	21.2 (18.9-24.2)	<0.0001 [A]
School education (years) [c]	15 (2)	15 (12-18)	14 (2)	14 (12-17)	15 (2)	15 (12-18)	15 (2)	15 (12-18)	15 (2)	15 (12-18)	<0.0001 [A]
Ejaculation abstinence (hours) [d]	78 (36)	65 (50-136)	76 (32)	64 (49-136)	77 (35)	64 (50-136)	82(51)	67 (51-138)	78 (28)	66 (51-136)	0.0002 [A]
		Frequency (%)		Frequency (%)		Frequency (%)		Frequency (%)		Frequency (%)	
Have (had)											
Cryptorchidism [e]		8.2		6.9		8.3		12.7		6.3	0.02 [B]
Testicular torsion		0.1		0.2		0.0		0.3		0.0	0.5 [B]
Orchitis		1.4		1.6		1.7		1.0		1.0	0.8 [B]
Varicocele		0.1		0,0		0.3		0.3		0.0	0.2 [B]
Inguinal hernia		1.1		0.7		2.3		1.0		1.0	0.2 [B]
STD [f]		2.1		2.8		1.0		1.3		2.3	0.3 [B]
Thyroid disease or diabetes		0.1		0.2		0.3		0,0		0.0	0.7 [B]
Taken medicine [g]		10.4		13.7		8.0		8.7		7.3	0.0006 [B]
Caused pregnancy		3.8		4.4		2.7		3.3		4.0	0.6 [B]
Experienced fertility problem [h]		0.9		1.8		0.3		0.0		0.7	0.03 [B]
Varicocele diagnosed in study [i]		27.1		26.8		24.3		20.0		37.5	<0.0001 [B]
Tobacco smoker		49.6		62.4		34.6		42.8		45.0	<0.0001 [B]
Exposed to tobacco in utero [j]		2,0		2.5		1.3		1.3		2.3	0.6 [B]

SD: Standard deviation.
(5-95): 5-95th percentile.
a: Size assessed by palpation. Two men had non-palpable left testicles, one from Kawasaki and one from Kanazawa due to previous orchidectomy because of testiuclar torsion.
b: Age calculated as difference between day of attendance in study and selfreported day of birth.
c: All participants were university students.
d: Ejaculation abstinence period calculated as difference between time of current ejaculation and self-reported time of previous ejaculation.
e: Not born with both testicles in scrotum (irrespective of spontaneous descend or treatment).
f: Diagnosed with epididymitis, chlamydia or gonnorhoea.
g: Taken any medication recent 3 months prior to participation in study. For 93%, 61%, 61% and 60% from Kawasaki, Osaka, Kanazawa and Nagasaki, respectively it was against either infection, allergy or pain.
h: Have had unprotected intercourse without causing a pregnancy during a 12 months period.
i: Varicocele diagnosed during this study, irrespective of previous self-reported information.
j: In utero exposed to maternal tobacco smoking.

A: Kruskal-Wallis test
B: Fisher's exact test

Table 2: Semen quality of young men from four cities in Japan.

	Observed		Adjusted
	Mean (SD)	Median (5-95)	Median (95%CI)
Semen volume (ml)			
Entire study population	2.9 (1.4)	2.7 (1.0-5.5)	3.0 (2.8-3.2)
Kawasaki	2.7 (1.3)	2.5 (0.9-5.2)	2.8 (2.7-3.2)
Osaka	2.9 (1.4)	2.7 (1.0-5.6)	2.9 (2.7-3.2)
Kanazawa	3.3 (1.5)	3.0 (1.2-6.4)	3.3 (3.1-3.6)
Nagasaki	2.9 (1.4)	2.8 (0.9-5.4)	3.0 (2.8-3.2)
p-value			p=0.0006
Sperm concentration (mill/ml)			
Entire study population	73 (58)	59 (10-185)	59 (52-68)
Kawasaki	71 (61)	55 (9-185)	57 (48-66)
Osaka	75 (61)	60 (9-195)	61 (50-74)
Kanazawa	72 (55)	60 (7-183)	61 (51-73)
Nagasaki	76 (54)	64 (12-181)	61 (51-74)
p-value			p=0.138
Total sperm count (mill)			
Entire study population	201 (183)	159 (18-509)	177 (153-206)
Kawasaki	185 (193)	143 (17-472)	161 (135-191)
Osaka	202 (178)	163 (28-508)	179 (146-221)
Kanazawa	228 (184)	185 (15-546)	201 (165-246)
Nagasaki	201 (161)	166 (22-531)	183 (150-224)
p-value			p=0.002
Motile spermatozoa (%)			
Entire study population	67 (14)	69 (42-88)	67 (65-69)
Kawasaki	66 (14)	68 (40-87)	65 (62-67)
Osaka	67 (12)	67 (52-84)	64 (61-67)
Kanazawa	62 (14)	64 (38-82)	60 (57-63)
Nagasaki	76 (14)	78 (48-93)	75 (73-78)
p-value			p<0.0001
Morphologically normal spermatozoa (%) [a]			
Entire study population	10.3 (6.0)	9 (2.5-21.5)	9.9 (8.8-10.9)
Kawasaki	10.5 (6.2)	9 (2.5-23)	9.3 (7.9-10.6)
Osaka	9.2 (5.2)	8.5 (2.5-19)	8.5 (7.2-9.8)
Kanazawa	10.0 (6.1)	8.8 (2-21)	9.3 (8.0-10.5)
Nagasaki	11.7 (6.1)	11 (3-23)	11.2 (10.0-12.4)
p-value			p<0.0001

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis.
Semen volume, sperm concentration and total sperm counts adjusted to a period of ejaculation abstinence of 96 h for a 21 years-old-man
Motility and morphology adjusted for a 21 years-old-man, winter season
See text for further explanation.
p-value: Based on regression analyses of cubic root transformed values, comparing all four groups.
a: Morphology results only available for 869 men.

Table 3: Reproductive hormone levels of young men from four cities in Japan

	Observed		Adjusted
	Mean (SD)	Median (5-95)	Median (95%CI)
FSH (U/l)			
Entire study population	2.5 (1.3)	2.2 (1.1-4.9)	2.3 (2.2-2.5)
Kawasaki	2.7 (1.3)	2.4 (1.1-5.2)	2.4 (2.3-2.6)
Osaka	2.5 (1.3)	2.2 (1.1-5.0)	2.3 (2.1-2.4)
Kanazawa	2.3 (1.3)	2.1 (1-4.4)	2.1 (1.9-2.3)
Nagasaki	2.3 (1.2)	2.1 (0.9-4.4)	2.1 (1.9-2.2)
p-value			p<0.0001
Inhibin-B (pg/ml)			
Entire study population	202 (64)	197 (110-314)	190 (181-199)
Kawasaki	207 (64)	202 (113-322)	191 (181-201)
Osaka	214 (67)	210 (117-336)	193 (182-205)
Kanazawa	191 (62)	190 (104-304)	177 (164-191)
Nagasaki	188 (60)	182 (103-286)	176 (165-187)
p-value			p=0.003
LH (U/l)			
Entire study population	3.2 (1.3)	2.9 (1.5-5.6)	2.7 (2.6-2.9)
Kawasaki	3 (1.3)	2.8 (1.4-5.3)	2.7 (2.5-2.8)
Osaka	3.5 (1.4)	3.2 (1.8-6.4)	3.0 (2.9-3.2)
Kanazawa	3.3 (1.4)	3.1 (1.6-5.8)	2.8 (2.6-3.1)
Nagasaki	2.9 (1.0)	2.8 (1.5-4.9)	2.6 (2.4-2.8)
p-value			p<0.0001
Testosterone (nmol/l)			
Entire study population	25 (8)	24 (14-39)	26 (25-27)
Kawasaki	26 (7)	25 (15-39)	26 (25-27)
Osaka	27 (9)	26 (15-42)	27 (26-28)
Kanazawa	22 (7)	21 (12-35)	23 (21-24)
Nagasaki	22 (7)	21 (13-36)	23 (21-24)
p-value			p<0.0001
SHBG (nmol/l)			
Entire study population	28 (11)	26 (13-46)	27 (26-28)
Kawasaki	30 (11)	29 (14-49)	28 (27-30)
Osaka	29 (11)	27 (14-50)	26 (25-28)
Kanazawa	24 (9)	23 (12-41)	23 (21-24)
Nagasaki	24 (9)	23 (12-42)	23 (21-24)
p-value			p<0.0001
cFT (pmol/l) [a]			
Entire study population	607 (192)	585 (326-949)	638 (613-663)
Kawasaki	619 (181)	605 (339-924)	630 (604-657)
Osaka	672 (214)	649 (371-1029)	675 (642-710)
Kanazawa	556 (183)	527 (298-894)	589 (552-628)
Nagasaki	566 (177)	550 (316-922)	583 (552-615)
p-value			p<0.0001

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis, adjusted to blood sampling at 10:00 am in winter season, representing af 21 years-old man having a BMI of 21.
p-value: Based on regression analyses of natural logarithmic transformed values, comparing all four groups.
a: Calculated free-testosterone.

Ethics

This study got the approval of the Ethics Review Board in each university and hospital. All participants gave their written consent before participating in the study.

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Data sharing statement

There is no additional data available.

Contributor statement

NJ, NES, JT, SN, MNM, MY and TI conceived and designed this study.

Acquisition of data: TI, MY, SN, KY, KB, MN, EK, JK, AO, KM, AT, HK, and JE.

Data analysis was performed by MNM, NJ and TI.

NJ, TI, SN and JT drafted the manuscript.

MV assessed the sperm morphology smears.

JK, EK, MN, KB, KY, AT, KM, AO, JE, HK and TI performed the physical examinations and collected the data.

All authors participated in the interpretation of data.

- Revising the article critically for important intellectual content: All authors.
- Final approval of the version to be published: All authors.

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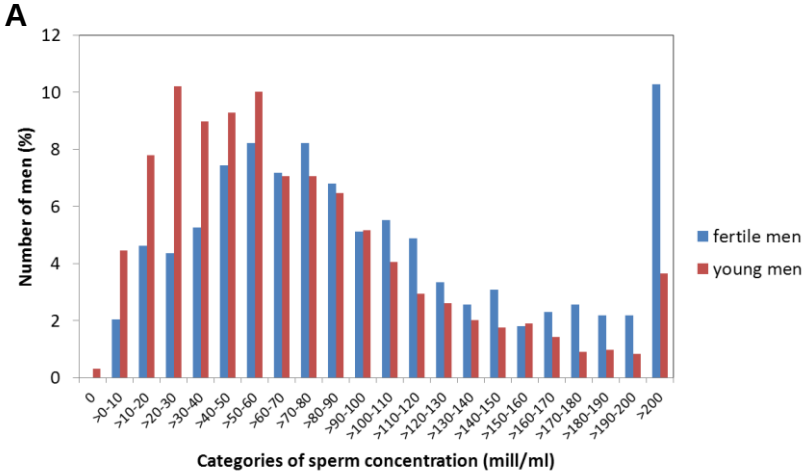
A



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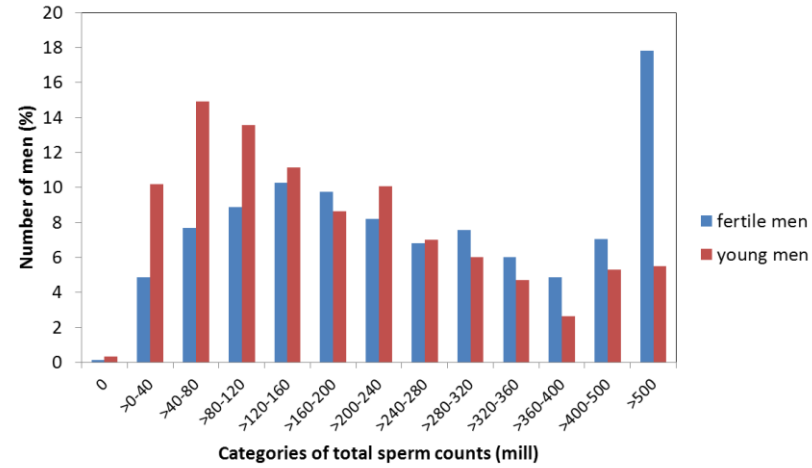

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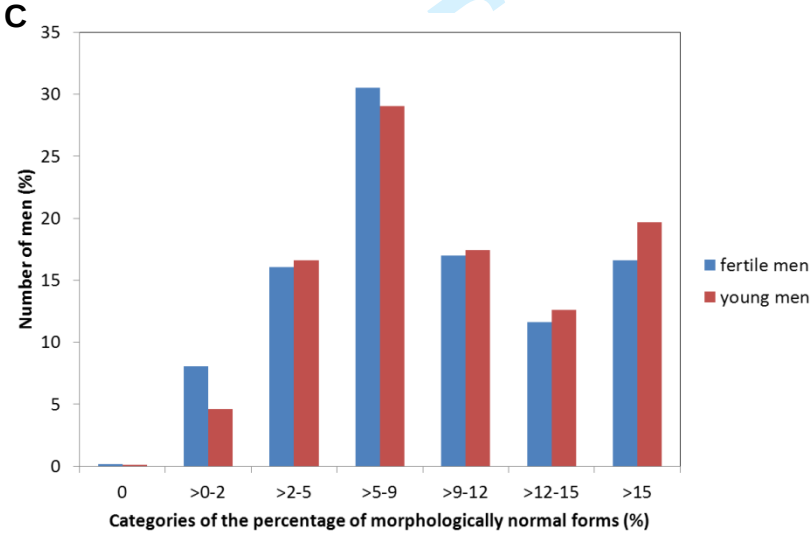

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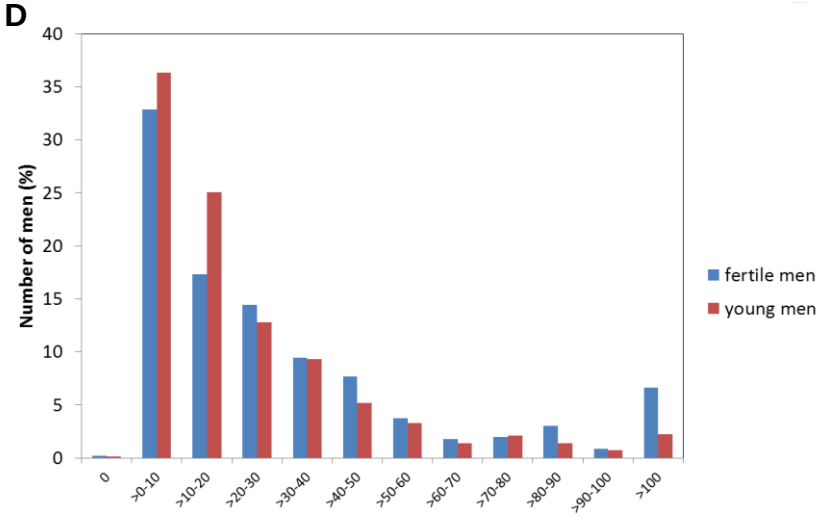

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cross-sectional studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 4
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3, 4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-5
Objectives	3	State specific objectives, including any prespecified hypotheses	4, 5
Methods			
Study design	4	Present key elements of study design early in the paper	4, 5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5, 6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	5, 6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	3, 7, 8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8
Bias	9	Describe any efforts to address potential sources of bias	8, 9
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7-10
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-10
		(*b*) Describe any methods used to examine subgroups and interactions	8, 9
		(*c*) Explain how missing data were addressed	N/A
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	5, 6
		(*e*) Describe any sensitivity analyses	7
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 6
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	6, 16 (Table 1)
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers of outcome events or summary measures	10-12, 17, 18 (Table 2 and 3)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10-12, 17, 18 (Table 2 and 3)
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	3, 4, 12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14, 15
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Semen quality of 1,559 young men from four cities in Japan

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Title:

Semen quality of 1,559 young men from four cities in Japan

Study design:

Cross sectional population-based study

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Semen quality and reproductive hormones in Japanese young men.

ARTICLE SUMMARY

Article focus:

- There has only been one Japanese study reporting semen quality of presumably normal young men indicating good semen quality compared with men from Europe.
- To establish the frequency of impaired semen quality among normal young Japanese men.

Key messages:

- Semen quality of the young men was significantly poorer than that of partners of pregnant women, and 32% may have reduced fertility chances.
- Semen quality was highly variable.
- These results will serve as a reference for future studies on time trends in semen quality in Japan and for comparison with future studies of university students in other countries.

Strengths and limitations:

- Large-scale prospective study of semen quality among Japanese young men.
- Standardised inclusion criteria and investigation procedures.
- Lack of influence of ethnicity and genetic background on semen quality.
- Relatively low participation rate limits the possibility to generalize the results for the whole population, which is the common problem for all semen studies

ABSTRACT

Objectives: To provide information of semen quality among normal young Japanese men and indicate the frequency of reduced semen quality.

Design: Cross-sectional, coordinated studies of Japanese young men included from university areas. The men had to be 18 to 24 years, and both the man and his mother had to be born in Japan. Background information was obtained from questionnaires. Standardized and quality controlled semen analyses were performed, reproductive hormones analysed centrally and results adjusted for confounding factors.

Setting: Four study centres in Japan (Kawasaki, Osaka, Kanazawa and Nagasaki).

Participants: 1,559 men, median age 21.1 years, included during 1999-2003.

Outcome measures: Semen volume, sperm concentration, total sperm count, sperm motility, sperm morphology and reproductive hormone levels.

Results: Median sperm concentration was 59 (95% confidence interval 52-68) million/mL, and 9.0% and 31.9% had less than 15 and 40 million/mL, respectively. Median percentage of morphologically normal spermatozoa was 9.6 (8.8-10.3) %. Small but statistically significant differences were detected for both semen and reproductive hormone variables between men from the four cities. Overall, the semen values were lower than those of a reference population of 792 fertile Japanese men.

Conclusions: Assuming the investigated men were representative for young Japanese men, a significant proportion of the population had suboptimal semen quality with reduced fertility potential, and as a group they had lower semen quality than fertile men. However, the definitive role – if any - of low semen quality for subfertility and low fertility rates remains to be investigated.

INTRODUCTION

Whether there has been a global decreasing trend in semen quality remains still controversial. In contrast, there is a consensus that regional differences exist.[1-7] Cross-sectional, coordinated studies of young men from the general populations have shown that men from the Western part of the Northern European countries have lower semen quality than men from the Eastern part or men from Southern Spain,[4-8] which is inversely correlated with the testicular cancer incidences.[8 9]

There has only been one Japanese study reporting semen quality of presumably normal young men[10] indicating good semen quality compared with men from Europe. However, the authors concluded that their results might be flawed by a selection bias and lack of ability to account for confounding factors, and they requested well-designed prospective studies to be performed in several regions of Japan.

Here, we present the results of prospectively designed, cross-sectional studies of young university students from four different provinces in Japan. Our objectives were to elucidate if reduced semen quality were frequent among Japanese men unselected for their fertility status, to examine possible regional differences, to provide a reference for future studies on time trends in semen quality, and to compare the results with those obtained in other countries.

METHODS

The investigations took place at four study centres based in departments of urology at university hospitals in Kawasaki, Osaka, Kanazawa and Nagasaki in Japan. The investigation procedures described below were the same as those of the previously published European studies[4 5 7] except for the selection of study populations and assessment of semen volume.

Study populations of young men

University students were informed about the study through posters placed in several conspicuous places on the campuses of the universities connected to the four study centres.

Serially numbered leaflets giving detailed information about the study were attached to the posters. A candidate volunteer had to call the study centre, inform the serial number printed on his copy of the leaflet to make an appointment for the investigations approximately one week later, and to receive a package of documents within days by mail. The package included further written information, a questionnaire and the instruction to preferably abstain from ejaculation for at least 48 hours prior to producing the semen sample for the study. Further inclusion criteria were that the man was 18-24 years, and that both he and his mother were born in Japan. Failure to comply with the request for ejaculation abstinence period was not a reason for exclusion, but the abstinence time was recorded according to the information given by the study subjects at the time of the semen sample delivery. On the day of attendance that was set at a certain time in the morning, the man returned the completed questionnaire, underwent a physical examination, provided a semen sample and had a blood sample drawn.

The study in Kawasaki covered two separate periods: May 1999 to May 2000 and April 2002 to May 2003, and the study periods in Osaka, Kanazawa, and Nagasaki were September 2002 to October 2003, July 2002 to June 2003, and July 2002 to July 2003, respectively. In total 9,374 leaflets were taken by the students and 1,559 (16.6%) participated; 14.5% (658/4,534) from Kawasaki, 11.7% (300/2,570) from Osaka, 21.9% (300/1,371) from Kanazawa, and 33.3% (301/899) from Nagasaki.

The healthy subgroup (n=1,307) of the entire study population (n=1,559), who had no history of cryptorchidism, testicular torsion, orchitis, varicocele, inguinal hernia, caused pregnancy, and experienced fertility problem, was examined separately (Supplemental Table 1).

Questionnaires

The questionnaire included information on age and previous or current diseases, including any known history of fertility. To assure the quality of the information regarding previous conditions the men were asked to fill in the questionnaire – if possible – in collaboration with their parents.

Physical examination

The participants had their testes size measured by use of a Prader orchidometer (Pharmacia & Upjohn, Copenhagen, Denmark). The presence of varicocele or other scrotal abnormalities and the Tanner stage of pubic hair were evaluated. Body weight and height were self-reported, and body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters.

Semen samples

The ejaculation abstinence period was calculated as the time between the current and previous ejaculation based on self-reported information from the men. Semen samples were collected at the laboratory and kept at 37°C during liquefaction. Semen volume was assessed by aspirating the entire sample into a graduated 5ml syringe (TERUMO, Tokyo). Sperm motility was assessed on 10 µl of well-mixed semen placed on a clean glass slide, covered with a 22x22 mm coverslip, and then examined at a total magnification of 400 times on the heating stage at 37°C of a microscope. The sperm were classified as either motile (WHO motility classes A, B or C) or immotile (class D), in order to record the proportion of motile sperm.[11] The motility assessment was repeated on a second 10 µl aliquot of semen, and the average value of the two samples was calculated. For the assessment of sperm concentration the samples were diluted in a solution of 0.6 mol/L $NaHCO_3$ and 0.4% (v/v) formaldehyde in distilled water, subsequently assessed using Bürker-Türk haemocytometers. Only sperm with tails were counted. Smears were prepared for morphological evaluation, Papanicolaou stained and finally assessed according to strict criteria[12] by one examiner (MV) in Finland between 2009 and 2010.

Quality control of sperm concentration assessment

Inter-laboratory variation in assessment of sperm concentration was monitored by an external quality control (QC) programme coordinated by the Department of Growth and Reproduction Copenhagen, Denmark[3 4] during the study period.

Blood samples

A blood sample was drawn from a cubital vein of each participant usually in the morning to reduce the effect of diurnal variation in hormone levels, and the serum was separated by centrifugation after clotting and stored at –20°C. The frozen serum was sent to the Department of Growth and Reproduction, Rigshospitalet, in Copenhagen, Denmark for a centralised hormone analysis. Levels of testosterone, FSH, LH and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). Inhibin-B was measured by a specific two-sided enzyme immunometric assay (Serotec, UK). Intra- and inter-assay coefficients of variations (CV) for measurements of both FSH and LH were 3% and 4.5%, respectively. CVs for both testosterone and SGBG were <8% and <5%, respectively. The intra- and inter-assay CVs for inhibin-B were 15% and 18%. Free testosterone (cFT) was calculated from total testosterone and SHBG using fixed albumin level of 43.8 g/L as described by Vermeulen.[13]

Comparison population: Fertile men (partners of pregnant women)

From January 1999 to February 2002, our group also examined the semen quality of 792 fertile men (partners of pregnant women) (a manuscript describing these have been submitted in parallel). Participation of these men was similar to that of current study population of young men: they answered a questionnaire, delivered a semen sample, and had a physical examination performed. The results of their semen analyses were used for comparison to that of the young men.

Statistical analysis

Standard statistics (mean, median, standard deviation (SD), 5-95 percentiles and frequencies) were used for description (Tables 1-3). Between-group differences for continuous variables giving the basic description of the study population were tested by the non-parametric Kruskal-Wallis test. Between-group differences for categorical variables were tested with the Fisher's exact test.

The main outcome variables were the assessed semen and hormone variables, and the between group differences were tested by multiple linear regression (Tables 2 and 3). Semen volume, sperm concentration and total sperm counts were best normalised by cubic root transformation before analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa were logit-transformed. Percentages of morphologically normal spermatozoa entered the model untransformed. Ejaculation abstinence up to 96 hours had a linear increasing effect and abstinence above 96 hours a slight, but significant non-linear increasing effect on semen volume, sperm concentrations and total sperm counts. Abstinence therefore entered the model as a covariate as linear splines and abstinence-squared for the part above 96 hours. For motility, winter season was associated with lower motility percentages and season was therefore included as a covariate. For all semen variables increasing age tended to be slightly, but negatively associated with semen variables and age was also included in the models. Duration from ejaculation to assessment was additionally evaluated as a confounder for motility, but found to be non-significant, and therefore not included.

Natural logarithmic transformation gave models in which differences between centres and effects of covariates are more easily interpretable. This alternative model approximate closely the model obtained by cubic root transformation and is used when reporting adjusted semen volumes, sperm concentrations and total sperm counts to represent a 21-year-old man having an ejaculation abstinence period of 96 hours. QC results did not show any significant inter-laboratory differences, changes during the study period or difference to the reference laboratory. Therefore, corrections of data were not needed to make them com-

parable. The logit-transformed motility data and untransformed morphology percentages were used to give adjusted levels for a 21-years-old man for these variables.

Differences with $p<0.05$ were considered statistically significant. All statistical analyses were done twice: MNM using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and NJ using PASW version 18.

RESULTS

A description of the study population is summarized in Table 1. Few men had caused a pregnancy or experienced infertility problems, in total 4.7%. During the preceding 3 months to participation in the study, 10.4% had used medications which were mainly antibiotics, painkillers, asthma or allergy medicines.

Semen results are shown in Table 2. “Observed” values are based on raw data, and “Adjusted” are the estimates from regression analyses taking covariates into account. Sperm concentrations did not differ between men from the four study centres, whereas the semen volume for men from Kanazawa was higher than in other centres ($p<0.0001$-0.02 in pair-wise comparisons). Consequently, also total sperm counts were higher for these men, but only significantly in the pair-wise comparison with men from Kawasaki ($p<0.02$). The percentages of motile spermatozoa differed significantly, because men from Nagasaki had higher frequencies of motile spermatozoa than men from other centres (adjusted medians 64-75%). The percentage of morphologically normal spermatozoa for men from Nagasaki was higher than that from Osaka ($p<0.0001$ in pair-wise comparison). These results were very similar to those from the healthy subgroup of young men (n=1,307: Supplemental Table 1) who had no history of reproductive problems, i.e. cryptorchidism, testicular torsion, orchitis, varicocele, inguinal hernia, STD, caused pregnancy, and experienced fertility problem.

Cumulated, 2.2% of men had a sperm concentration below 5 million/mL, 4.9% below 10 million/mL, 9.0% below 15 million/mL and 31.9% below 40 million/mL. For morphology 5.7% of men had normal spermatozoa below 5%. Figure 1 summarizes the distribution of

the sperm concentration, total sperm counts, percentages and total numbers of morphologically normal spermatozoa.

Figure 1 summarises the semen results of the young men in comparison to that of the 792 fertile men. The semen variables differed between these groups, with fertile men having higher semen volume ($p<0.002$), sperm concentration ($p<0.0001$), total sperm count ($p<0.0001$), total number of morphologically normal spermatozoa ($p<0.0001$)and the percentage of normal spermatozoa ($p=0.05$) than young men.

The semen variables of men whose mothers had smoked during pregnancy did not significantly differ from non-exposed men, but the number of smoking mother was very small. The men's own smoking or drinking habits (not shown in Table), previous experience of cryptorchidism, testicular torsion, orchitis, sexually transmitted diseases (chlamydia, gonnorhoea or epididymitis) or previous fertility experience did not affect their semen variables.

Reproductive hormone levels differed between the groups of young men (Table 3). Men from Kawasaki and Osaka had slightly higher FSH ($p<0.0001$-0.049) and inhibin-B ($p<0.003$-0.01) levels than men from Kanazawa and Nagasaki. A similar pattern was seen for total testosterone (all $p<0.0001$), SHBG (all $p<0.0001$) and cFT (all $p<0.0001$). The number of men with high (mean+2SD) gonadotropin levels was very small (1-4 men for FSH 0.3-1.3%, and 2-14 for LH 0.7-4.7%). T/LH ratio of men from Kawasaki, Osaka, Kanazawa, and Nagawaki were 9.74±4.26, 8.75±3.54, 8.36±3.76, and 7.45±3.51, respectively. All pair-wise comparisons between centres except Osaka vs. Nagasaki were statistically significant ($p<0.0001$-0.01).

For the entire group of men the median (5-95 percentiles) sizes of the left and right testes were both 22 ml (14-28 ml). The average testis size (mean of left and right) differed significantly between the study groups, ranging from 19 to 23 ml in medians (Table 1). Overall 98.8% of study subjects had a pubic hair distribution of Tanner stage 4 or higher: Kawasaki 98.2%, Osaka 98.0%, Kanazawa 100% and Nagasaki 99.7%.

During the physical examination 27.1% of men was diagnosed with a varicocele; 14.3% stage 1, 9.1% stage 2, and 3.7% stage 3. Varicocele was on the left side only

in23.6%, both sides in 2.9% and only on the right side in 0.6% of men. The presence of a varicocele was non-significantly associated with an 8% (95% confidence interval –20%;+6%) reduction in sperm concentration and 11% decline (-23%;+4%) in total sperm count. No tendencies could be detected for sperm motility or morphology. Inhibin-B and total testosterone tended to be lower in men having a varicocele, however, non-significantly ($p>0.05$).

DISCUSSION

Sperm concentration, total sperm count, percentage of morphologically normal spermatozoa and percentage of motile spermatozoa varied tremendously between individual participants. However, only small but statistically significant differences were detected for both semen and reproductive hormone levels between young men from the four provinces in Japan. Thus, from a biological point of view these groups of men can be regarded as similar. As expected, semen quality of the young men was significantly poorer than that of partners of pregnant women.

To our knowledge, this is the first large-scale prospectively designed study to explore potential differences in testicular function parameters in Japanese men that were not selected by any fertility status. The men were enrolled by the same type of advertisement. They were all university students. We tested for effects of various covariates and accounted for these when necessary. Thus, the comparison between the four groups is valid, and the results may serve as a reference for future studies on time trends in semen quality.

In Japan, there is not a compulsory medical examination of young men as in the Northern-European countries,[4 7] which makes it difficult to recruit men that are representative for the general population. Itoh *et al* [10] detected a median sperm concentration of 81 million/mL in 207 young men (18-22 years old) examined in 1998 in Sapporo but also concluded that selection bias in the recruitment and variable ejaculation abstinence might have affected the results. In the design of our study, we therefore decided to restrict the invitation to university students to get a well-characterized study population. Men who have experi-

enced fertility problems may be more likely to volunteer for semen studies than men without any problems.[14] Since the sperm count cannot be known without laboratory analysis, it is unlikely that any of the volunteers would have had such information. Testicular size might hint to fertility problem if it were very small. However, there was only small difference (slightly larger in the present cohort) between the testicular size of the young and the fertile men[15] in Japan. Moreover, semen variables of the healthy subgroup of young men (n=1,307) who had no history of reproductive problems were very similar to those of the entire study population (n=1,559). Likewise, it should be noted that the incidence of cryptorchidism was considerably high, which could be an indicator of recruitment bias. In the present study, however, it is unlikely to be a recruitment bias, because the information about cryptorchidism was based on the questionnaire data, i.e. history of cryptorchidism, not current cryptorchidism. This included cryptorchidism at any time, i.e. both congenital and acquired, which may have different causes and consequences. In fact, there were no differences in semen quality between the healthy subgroup of young men (Supplemental Table 1) and the entire study population (Table 2) including the men with history of cryptorchidism or other reproductive problems. We restricted the age of participants to 18-24 years assuming that the majority in such a group would not yet have any direct knowledge about their own fertility chances, since the average Japanese man fathers his first child at the age of 31.8 years.[16] Less than 1% of the participants had experienced fertility problems, and such problems are therefore unlikely to severely bias the obtained results. Poorly educated men may be more likely to refuse participation in studies that require delivery of semen samples.[17] However, higher educational level has been positively associated with semen quality.[17 18] Thus, the selection of university students may bias the results towards a higher level. Nevertheless, semen variables of the healthy subgroup of young men (Supplemental Table 1) who had no history of reproductive problems were very similar to those of the entire study population (Table 2). We thus considered that semen results of the present study population was unlikely to have been subjected to a significant bias.

There were small differences in inhibin-B levels that were lower in men from Kanazawa and Nagasaki than in men from Kawasaki and Osaka. Similar trends were detected for total testosterone, SHBG, and cFT. The slightly higher inhibin-B levels in men from Kawasaki and Osaka could theoretically indicate a higher spermatogenic activity in these, which however does not fit with the sperm count findings. Also, we hesitate to draw such a conclusion since the detected inhibin-B concentrations at this level do not correlate strongly with sperm counts.[19] We have no explanation for the higher total testosterone in men from Kawasaki and Osaka compared to other centres. It is important to keep in mind that the adjusted hormone levels in Table 3 were adjusted for the effect of BMI, which thus does not explain the findings. T/LH ratio may inform about Leydig cell function, and therefore we also analysed it. Variation between the study centres was small but statistically significant; however, biological significance of variation in this scale is questionable.

Diagnostic accuracy of varicocele is very dependent on the clinical experience of the investigator. The high frequency of varicocele may reflect this, since the majority of the cases were grade 1. We saw no significant negative effect on semen quality, which however, is likely due to the low grade in most varicoceles.

In the European studies semen volume was assessed by weighing which now is the "gold standard", because aspiration – as used in our study – underestimates the volume by approximately 0.4 mL.[20 21] Thus, the true median semen volume might have been 3.4 mL, which is closer to the volumes reported from Finland, Denmark, Germany, and Spain,[3 7 8] and thus the adjusted median total sperm count in our study would have been 200 million rather than 177. Inter-observer variation may explain the higher semen volume detected in men from Kanazawa compared to other centres, because volume measurements were not under external quality control.

Three Chinese studies have described semen qualities in apparently normal men. Healthy men, 20-60 years of age, had a median sperm concentration and total sperm count of approximately 65 million/mL and 154 million, respectively.[22] The youngest age group, 20-25 years old, comprised only 6.1% of the study population, and overall 83% had previ-

ously fathered a child. Another study investigated 20-40 years old men but excluded those with known andrological diseases and reported a median sperm concentration and total sperm count of 78 million/mL and 168 million.[23] Junqing *et al*[24] detected a mean sperm concentration and total sperm count of 55 million/mL and 124 million, respectively, in 22-30 years old men that underwent a premarital physical examination. Thus, the latter may be more comparable to ours, since the men presumably had little knowledge of their fertility potential. These Chinese reference values are clearly lower than the current Japanese figures, suggesting that there are regional differences in semen quality among Asian men. Interestingly, Chinese men who had at least a college education had lower sperm counts than men with a lower educational status.[24] If this were true also for Japanese men, the difference in semen quality between Japanese and Chinese men would be even greater than what is evident from the present results.

Other studies have shown an adverse effect of maternal smoking during pregnancy on the son's semen quality.[25-27] However, we did not find such an effect, probably because the number of smoking mothers was very small. By contrast, the smoking rates of the young men themselves looks exceedingly high for a developed nation, but this is a common trend in Japanese male, which is consistent with the figures from National Health and Nutrition Survey (47.4% in total and 60.8% in 20-29 age group in 2000)[28], as well as the results from the previous our study of the fertile Japanese men (52.8% in total).[15]

The Japanese men appeared to have higher sperm counts than men from the Northern Europe[4 5 7 29-32] but slightly lower than men from Spain.[8] Thus, Japanese men may be ranked as having better sperm counts than many populations of European young men. Nevertheless, this does not imply that impaired semen quality is not a problem among young Japanese men. Nearly 10% had sperm counts below the current WHO reference levels of 15 million/mL or 39 million[32] rendering them for high risk of reduced fertility in the future. Nearly one third of men had sperm concentration less than 40 million/mL, indicating reduced fecundity.[34-37] We are therefore concerned that a significant proportion of the men may experience problems when they reach the ages when they want to reproduce.[34-37]

Our prospectively designed study showed no overall difference between men from the four investigation sites, and the semen results were similar to those previously reported for Finnish men that until now have been considered to have the best semen quality among young men from the Northern Europe. The study cohorts were, however, not completely comparable. The recently detected adverse trends in semen quality and testis cancer incidences among men from Finland suggest underlying environmental causes.[32] Whether the semen quality of Japanese men has changed over the years cannot be answered by our current study, but a large proportion of investigated men were shown to have sub-optimal sperm counts. These results will serve as a reference for future studies on time trends in semen quality in Japan and for comparison with future studies of university students in other countries.

Table 1: Physical appearance and self-reported information of young men from four cities in Japan.

	Entire study population (n=1559)		Kawasaki (n=658)		Osaka (n=300)		Kanazawa (n=300)		Nagasaki (n=301)		
	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	p-values
Height (cms)	172 (6)	172 (163-181)	172 (6)	172 (163-182)	172 (6)	172 (163-181)	173 (6)	173 (163-181)	172 (5)	171 (163-180)	0.01 [A]
Weight (kgs)	64 (9)	63 (52-79)	64 (11)	63 (52-80)	63 (8)	63 (52-77)	64 (8)	63 (52-80)	63 (9)	62 (52-82)	0.4 [A]
BMI (kg/m2)	21.4 (2.7)	21.0 (18.2-26.0)	21.5 (3.0)	21.0 (18.2-26.0)	21.2 (2.2)	20.9 (18.2-25.4)	21.4 (2.4)	21.1 (18.4-25.8)	21.6 (2.6)	21.3 (18.0-26.2)	0.2 [A]
Mean of left and right size (ml) [a]	21 (4)	22 (14-28)	22 (4)	22 (15-28)	22 (5)	23 (14-29)	21 (4)	21 (14-29)	20 (4)	19 (13-26)	<0.0001 [A]
Age (years) [b]	21.3 (1.6)	21.1 (18.9-24.1)	20.8 (1.4)	20.7 (18.7-23.4)	21.7 (1.6)	21.6 (19.3-24.4)	21.8 (1.6)	21.9 (18.9-24.2)	21.3 (1.7)	21.2 (18.9-24.2)	<0.0001 [A]
School education (years) [c]	15 (2)	15 (12-18)	14 (2)	14 (12-17)	15 (2)	15 (12-18)	15 (2)	15 (12-18)	15 (2)	15 (12-18)	<0.0001 [A]
Ejaculation abstinence (hours) [d]	78 (36)	65 (50-136)	76 (32)	64 (49-136)	77 (35)	64 (50-136)	82(51)	67 (51-138)	78 (28)	66 (51-136)	0.0002 [A]
		Frequency (%)		Frequency (%)		Frequency (%)		Frequency (%)		Frequency (%)	
Have (had)											
Cryptorchidism [e]		8.2		6.9		8.3		12.7		6.3	0.02 [B]
Testicular torsion		0.1		0.2		0.0		0.3		0.0	0.5 [B]
Orchitis		1.4		1.6		1.7		1.0		1.0	0.8 [B]
Varicocele		0.1		0,0		0.3		0.3		0.0	0.2 [B]
Inguinal hernia		1.1		0.7		2.3		1.0		1.0	0.2 [B]
STD [f]		2.1		2.8		1.0		1.3		2.3	0.3 [B]
Thyroid disease or diabetes		0.1		0.2		0.3		0,0		0.0	0.7 [B]
Taken medicine [g]		10.4		13.7		8.0		8.7		7.3	0.0006 [B]
Caused pregnancy		3.8		4.4		2.7		3.3		4.0	0.6 [B]
Experienced fertility problem [h]		0.9		1.8		0.3		0.0		0.7	0.03 [B]
Varicocele diagnosed in study [i]		27.1		26.8		24.3		20.0		37.5	<0.0001 [B]
Tobacco smoker		49.6		62.4		34.6		42.8		45.0	<0.0001 [B]
Exposed to tobacco in utero [j]		2,0		2.5		1.3		1.3		2.3	0.6 [B]

SD: Standard deviation.
(5-95): 5-95th percentile.
a: Size assessed by palpation. Two men had non-palpable left testicles, one from Kawasaki and one from Kanazawa due to previous orchidectomy because of testiuclar torsion.
b: Age calculated as difference between day of attendance in study and selfreported day of birth.
c: All participants were university students.
d: Ejaculation abstinence period calculated as difference between time of current ejaculation and self-reported time of previous ejaculation.
e: Not born with both testicles in scrotum (irrespective of spontaneous descend or treatment).
f: Diagnosed with epididymitis, chlamydia or gonnorhoea.
g: Taken any medication recent 3 months prior to participation in study. For 93%, 61%, 61% and 60% from Kawasaki, Osaka, Kanazawa and Nagasaki, respectively it was against either infection, allergy or pain.
h: Have had unprotected intercourse without causing a pregnancy during a 12 months period.
i: Varicocele diagnosed during this study, irrespective of previous self-reported information.
j: In utero exposed to maternal tobacco smoking.

A: Kruskal-Wallis test
B: Fisher's exact test

Table 2: Semen quality of young men from four cities in Japan.

	Observed		Adjusted
	Mean (SD)	Median (5-95)	Median (95%CI)
Semen volume (ml)			
Entire study population	2.9 (1.4)	2.7 (1.0-5.5)	3.0 (2.8-3.2)
Kawasaki	2.7 (1.3)	2.5 (0.9-5.2)	2.8 (2.7-3.2)
Osaka	2.9 (1.4)	2.7 (1.0-5.6)	2.9 (2.7-3.2)
Kanazawa	3.3 (1.5)	3.0 (1.2-6.4)	3.3 (3.1-3.6)
Nagasaki	2.9 (1.4)	2.8 (0.9-5.4)	3.0 (2.8-3.2)
p-value			p=0.0006
Sperm concentration (mill/ml)			
Entire study population	73 (58)	59 (10-185)	59 (52-68)
Kawasaki	71 (61)	55 (9-185)	57 (48-66)
Osaka	75 (61)	60 (9-195)	61 (50-74)
Kanazawa	72 (55)	60 (7-183)	61 (51-73)
Nagasaki	76 (54)	64 (12-181)	61 (51-74)
p-value			p=0.138
Total sperm count (mill)			
Entire study population	201 (183)	159 (18-509)	177 (153-206)
Kawasaki	185 (193)	143 (17-472)	161 (135-191)
Osaka	202 (178)	163 (28-508)	179 (146-221)
Kanazawa	228 (184)	185 (15-546)	201 (165-246)
Nagasaki	201 (161)	166 (22-531)	183 (150-224)
p-value			p=0.002
Motile spermatozoa (%)			
Entire study population	67 (14)	69 (42-88)	67 (65-69)
Kawasaki	66 (14)	68 (40-87)	65 (62-67)
Osaka	67 (12)	67 (52-84)	64 (61-67)
Kanazawa	62 (14)	64 (38-82)	60 (57-63)
Nagasaki	76 (14)	78 (48-93)	75 (73-78)
p-value			p<0.0001
Morphologically normal spermatozoa (%) [a]			
Entire study population	10.3 (6.0)	9 (2.5-21.5)	9.9 (8.8-10.9)
Kawasaki	10.5 (6.2)	9 (2.5-23)	9.3 (7.9-10.6)
Osaka	9.2 (5.2)	8.5 (2.5-19)	8.5 (7.2-9.8)
Kanazawa	10.0 (6.1)	8.8 (2-21)	9.3 (8.0-10.5)
Nagasaki	11.7 (6.1)	11 (3-23)	11.2 (10.0-12.4)
p-value			p<0.0001

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis.
Semen volume, sperm concentration and total sperm counts adjusted to a period of ejaculation abstinence of 96 h for a 21 years-old-man
Motility and morphology adjusted for a 21 years-old-man, winter season
See text for further explanation.
p-value: Based on regression analyses of cubic root transformed values, comparing all four groups.
a: Morphology results only available for 869 men.

Table 3: Reproductive hormone levels of young men from four cities in Japan

	Observed		Adjusted
	Mean (SD)	**Median (5-95)**	**Median (95%CI)**
FSH (U/l)			
Entire study population	2.5 (1.3)	2.2 (1.1-4.9)	2.3 (2.2-2.5)
Kawasaki	2.7 (1.3)	2.4 (1.1-5.2)	2.4 (2.3-2.6)
Osaka	2.5 (1.3)	2.2 (1.1-5.0)	2.3 (2.1-2.4)
Kanazawa	2.3 (1.3)	2.1 (1-4.4)	2.1 (1.9-2.3)
Nagasaki	2.3 (1.2)	2.1 (0.9-4.4)	2.1 (1.9-2.2)
p-value			p<0.0001
Inhibin-B (pg/ml)			
Entire study population	202 (64)	197 (110-314)	190 (181-199)
Kawasaki	207 (64)	202 (113-322)	191 (181-201)
Osaka	214 (67)	210 (117-336)	193 (182-205)
Kanazawa	191 (62)	190 (104-304)	177 (164-191)
Nagasaki	188 (60)	182 (103-286)	176 (165-187)
p-value			p=0.003
LH (U/l)			
Entire study population	3.2 (1.3)	2.9 (1.5-5.6)	2.7 (2.6-2.9)
Kawasaki	3 (1.3)	2.8 (1.4-5.3)	2.7 (2.5-2.8)
Osaka	3.5 (1.4)	3.2 (1.8-6.4)	3.0 (2.9-3.2)
Kanazawa	3.3 (1.4)	3.1 (1.6-5.8)	2.8 (2.6-3.1)
Nagasaki	2.9 (1.0)	2.8 (1.5-4.9)	2.6 (2.4-2.8)
p-value			p<0.0001
Testosterone (nmol/l)			
Entire study population	25 (8)	24 (14-39)	26 (25-27)
Kawasaki	26 (7)	25 (15-39)	26 (25-27)
Osaka	27 (9)	26 (15-42)	27 (26-28)
Kanazawa	22 (7)	21 (12-35)	23 (21-24)
Nagasaki	22 (7)	21 (13-36)	23 (21-24)
p-value			p<0.0001
SHBG (nmol/l)			
Entire study population	28 (11)	26 (13-46)	27 (26-28)
Kawasaki	30 (11)	29 (14-49)	28 (27-30)
Osaka	29 (11)	27 (14-50)	26 (25-28)
Kanazawa	24 (9)	23 (12-41)	23 (21-24)
Nagasaki	24 (9)	23 (12-42)	23 (21-24)
p-value			p<0.0001
cFT (pmol/l) [a]			
Entire study population	607 (192)	585 (326-949)	638 (613-663)
Kawasaki	619 (181)	605 (339-924)	630 (604-657)
Osaka	672 (214)	649 (371-1029)	675 (642-710)
Kanazawa	556 (183)	527 (298-894)	589 (552-628)
Nagasaki	566 (177)	550 (316-922)	583 (552-615)
p-value			p<0.0001

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis, adjusted to blood sampling at 10:00 am in winter season, representing af 21 years-old man having a BMI of 21.
p-value: Based on regression analyses of natural logarithmic transformed values, comparing all four groups.
a: Calculated free-testosterone.

Ethics

This study got the approval of the Ethics Review Board in each university and hospital. All participants gave their written consent before participating in the study.

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Data sharing statement

There is no additional data available.

Contributor statement

NJ, NES, JT, SN, MNM, MY and TI conceived and designed this study.

Acquisition of data: TI, MY, SN, KY, KB, MN, EK, JK, AO, KM, AT, HK, and JE.

Data analysis was performed by MNM, NJ and TI.

NJ, TI, SN and JT drafted the manuscript.

MV assessed the sperm morphology smears.

JK, EK, MN, KB, KY, AT, KM, AO, JE, HK and TI performed the physical examinations and collected the data.

All authors participated in the interpretation of data.

- Revising the article critically for important intellectual content: All authors.
- Final approval of the version to be published: All authors.

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Title:

Semen quality of 1,559 young men from four cities in Japan

Study design:

Cross sectional population-based study

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Semen quality and reproductive hormones in Japanese young men.

ARTICLE SUMMARY

Article focus:

- There has only been one Japanese study reporting semen quality of presumably normal young men indicating good semen quality compared with men from Europe.
- To establish the frequency of impaired semen quality among normal young Japanese men.

Key messages:

- Semen quality of the young men was significantly poorer than that of partners of pregnant women, and 32% may have reduced fertility chances.
- Semen quality was highly variable.
- These results will serve as a reference for future studies on time trends in semen quality in Japan and for comparison with future studies of university students in other countries.

Strengths and limitations:

- Large-scale prospective study of semen quality among Japanese young men.
- Standardised inclusion criteria and investigation procedures.
- Lack of influence of ethnicity and genetic background on semen quality.
- Relatively low participation rate limits the possibility to generalize the results for the whole population, which is the common problem for all semen studies

ABSTRACT

Objectives: To provide information of semen quality among normal young Japanese men and indicate the frequency of reduced semen quality.

Design: Cross-sectional, coordinated studies of Japanese young men included from university areas. The men had to be 18 to 24 years, and both the man and his mother had to be born in Japan. Background information was obtained from questionnaires. Standardized and quality controlled semen analyses were performed, reproductive hormones analysed centrally and results adjusted for confounding factors.

Setting: Four study centres in Japan (Kawasaki, Osaka, Kanazawa and Nagasaki).

Participants: 1,559 men, median age 21.1 years, included during 1999-2003.

Outcome measures: Semen volume, sperm concentration, total sperm count, sperm motility, sperm morphology and reproductive hormone levels.

Results: Median sperm concentration was 59 (95% confidence interval 52-68) million/mL, and 9.0% and 31.9% had less than 15 and 40 million/mL, respectively. Median percentage of morphologically normal spermatozoa was 9.6 (8.8-10.3) %. Small but statistically significant differences were detected for both semen and reproductive hormone variables between men from the four cities. Overall, the semen values were lower than those of a reference population of 792 fertile Japanese men.

Conclusions: Assuming the investigated men were representative for young Japanese men, a significant proportion of the population had suboptimal semen quality with reduced fertility potential, and as a group they had lower semen quality than fertile men. However, the definitive role – if any - of low semen quality for subfertility and low fertility rates remains to be investigated.

INTRODUCTION

Whether there has been a global decreasing trend in semen quality remains still controversial. In contrast, there is a consensus that regional differences exist.[1-7] Cross-sectional, coordinated studies of young men from the general populations have shown that men from the Western part of the Northern European countries have lower semen quality than men from the Eastern part or men from Southern Spain,[4-8] which is inversely correlated with the testicular cancer incidences.[8 9]

There has only been one Japanese study reporting semen quality of presumably normal young men[10] indicating good semen quality compared with men from Europe. However, the authors concluded that their results might be flawed by a selection bias and lack of ability to account for confounding factors, and they requested well-designed prospective studies to be performed in several regions of Japan.

Here, we present the results of prospectively designed, cross-sectional studies of young university students from four different provinces in Japan. Our objectives were to elucidate if reduced semen quality were frequent among Japanese men unselected for their fertility status, to examine possible regional differences, to provide a reference for future studies on time trends in semen quality, and to compare the results with those obtained in other countries.

METHODS

The investigations took place at four study centres based in departments of urology at university hospitals in Kawasaki, Osaka, Kanazawa and Nagasaki in Japan. The investigation procedures described below were the same as those of the previously published European studies[4 5 7] except for the selection of study populations and assessment of semen volume.

Study populations of young men

University students were informed about the study through posters placed in several conspicuous places on the campuses of the universities connected to the four study centres.

Serially numbered leaflets giving detailed information about the study were attached to the posters. A candidate volunteer had to call the study centre, inform the serial number printed on his copy of the leaflet to make an appointment for the investigations approximately one week later, and to receive a package of documents within days by mail. The package included further written information, a questionnaire and the instruction to preferably abstain from ejaculation for at least 48 hours prior to producing the semen sample for the study. Further inclusion criteria were that the man was 18-24 years, and that both he and his mother were born in Japan. Failure to comply with the request for ejaculation abstinence period was not a reason for exclusion, but the abstinence time was recorded according to the information given by the study subjects at the time of the semen sample delivery. On the day of attendance that was set at a certain time in the morning, the man returned the completed questionnaire, underwent a physical examination, provided a semen sample and had a blood sample drawn.

The study in Kawasaki covered two separate periods: May 1999 to ~~April~~ May 2000 and April 2002 to May 2003, and the study periods in Osaka, Kanazawa, and Nagasaki were September 2002 to October 2003, July 2002 to June 2003, and July 2002 to July 2003, respectively. In total 9,374 leaflets were taken by the students and 1,559 (16.6%) participated; 14.5% (658/4,534) from Kawasaki, 11.7% (300/2,570) from Osaka, 21.9% (300/1,371) from Kanazawa, and 33.3% (301/899) from Nagasaki.

The healthy subgroup (n=1,307) of the entire study population (n=1,559), who had no history of cryptorchidism, testicular torsion, orchitis, varicocele, inguinal hernia, caused pregnancy, and experienced fertility problem, was examined separately (Supplemental Table 1).

Questionnaires

The questionnaire included information on age and previous or current diseases, including any known history of fertility. To assure the quality of the information regarding previous

conditions the men were asked to fill in the questionnaire – if possible – in collaboration with their parents.

Physical examination

The participants had their testes size measured by use of a Prader orchidometer (Pharmacia & Upjohn, Copenhagen, Denmark). The presence of varicocele or other scrotal abnormalities and the Tanner stage of pubic hair were evaluated. Body weight and height were self-reported, and body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters.

Semen samples

The ejaculation abstinence period was calculated as the time between the current and previous ejaculation based on self-reported information from the men. Semen samples were collected at the laboratory and kept at 37°C during liquefaction. Semen volume was assessed by aspirating the entire sample into a graduated 5ml syringe (TERUMO, Tokyo). Sperm motility was assessed on 10 µl of well-mixed semen placed on a clean glass slide, covered with a 22x22 mm coverslip, and then examined at a total magnification of 400 times on the heating stage at 37°C of a microscope. The sperm were classified as either motile (WHO motility classes A, B or C) or immotile (class D), in order to record the proportion of motile sperm.[11] The motility assessment was repeated on a second 10 µl aliquot of semen, and the average value of the two samples was calculated. For the assessment of sperm concentration the samples were diluted in a solution of 0.6 mol/L $NaHCO_3$ and 0.4% (v/v) formaldehyde in distilled water, subsequently assessed using Bürker-Türk haemocytometers. Only sperm with tails were counted. Smears were prepared for morphological evaluation, Papanicolaou stained and finally assessed according to strict criteria[12] by one examiner (MV) in Finland between 2009 and 2010.

Quality control of sperm concentration assessment

Inter-laboratory variation in assessment of sperm concentration was monitored by an external quality control (QC) programme coordinated by the Department of Growth and Reproduction Copenhagen, Denmark[3 4] during the study period.

Blood samples

A blood sample was drawn from a cubital vein of each participant usually in the morning to reduce the effect of diurnal variation in hormone levels, and the serum was separated by centrifugation after clotting and stored at –20°C. The frozen serum was sent to the Department of Growth and Reproduction, Rigshospitalet, in Copenhagen, Denmark for a centralised hormone analysis. Levels of testosterone, FSH, LH and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). Inhibin-B was measured by a specific two-sided enzyme immunometric assay (Serotec, UK). Intra- and inter-assay coefficients of variations (CV) for measurements of both FSH and LH were 3% and 4.5%, respectively. CVs for both testosterone and SGBG were <8% and <5%, respectively. The intra- and inter-assay CVs for inhibin-B were 15% and 18%. Free testosterone (cFT) was calculated from total testosterone and SHBG using fixed albumin level of 43.8 g/L as described by Vermeulen.[13]

Comparison population: Fertile men (partners of pregnant women)

From January 1999 to February 2002, our group also examined the semen quality of 792 fertile men (partners of pregnant women) (a manuscript describing these have been submitted in parallel). Participation of these men was similar to that of current study population of young men: they answered a questionnaire, delivered a semen sample, and had a physical examination performed. The results of their semen analyses were used for comparison to that of the young men.

Statistical analysis

Standard statistics (mean, median, standard deviation (SD), 5-95 percentiles and frequencies) were used for description (Tables 1-3). Between-group differences for continuous variables giving the basic description of the study population were tested by the non-parametric Kruskal-Wallis test. Between-group differences for categorical variables were tested with the Fisher's exact test.

The main outcome variables were the assessed semen and hormone variables, and the between group differences were tested by multiple linear regression (Tables 2 and 3). Semen volume, sperm concentration and total sperm counts were best normalised by cubic root transformation before analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa were logit-transformed. Percentages of morphologically normal spermatozoa entered the model untransformed. Ejaculation abstinence up to 96 hours had a linear increasing effect and abstinence above 96 hours a slight, but significant non-linear increasing effect on semen volume, sperm concentrations and total sperm counts. Abstinence therefore entered the model as a covariate as linear splines and abstinence-squared for the part above 96 hours. For motility, winter season was associated with lower motility percentages and season was therefore included as a covariate. For all semen variables increasing age tended to be slightly, but negatively associated with semen variables and age was also included in the models. Duration from ejaculation to assessment was additionally evaluated as a confounder for motility, but found to be non-significant, and therefore not included.

Natural logarithmic transformation gave models in which differences between centres and effects of covariates are more easily interpretable. This alternative model approximate closely the model obtained by cubic root transformation and is used when reporting adjusted semen volumes, sperm concentrations and total sperm counts to represent a 21-year-old man having an ejaculation abstinence period of 96 hours. QC results did not show any significant inter-laboratory differences, changes during the study period or difference to the

reference laboratory. Therefore, corrections of data were not needed to make them comparable. The logit-transformed motility data and untransformed morphology percentages were used to give adjusted levels for a 21-years-old man for these variables.

Differences with $p<0.05$ were considered statistically significant. All statistical analyses were done twice: MNM using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and NJ using PASW version 18.

RESULTS

A description of the study population is summarized in Table 1. Few men had caused a pregnancy or experienced infertility problems, in total 4.7%. During the preceding 3 months to participation in the study, 10.4% had used medications which were mainly antibiotics, painkillers, asthma or allergy medicines.

Semen results are shown in Table 2. "Observed" values are based on raw data, and "Adjusted" are the estimates from regression analyses taking covariates into account. Sperm concentrations did not differ between men from the four study centres, whereas the semen volume for men from Kanazawa was higher than in other centres ($p<0.0001$-0.02 in pair-wise comparisons). Consequently, also total sperm counts were higher for these men, but only significantly in the pair-wise comparison with men from Kawasaki ($p<0.02$). The percentages of motile spermatozoa differed significantly, because men from Nagasaki had higher frequencies of motile spermatozoa than men from other centres (adjusted medians 64-75%). The percentage of morphologically normal spermatozoa for men from Nagasaki was higher than that from Osaka ($p<0.0001$ in pair-wise comparison). These results were very similar to those from the healthy subgroup of young men (n=1,307: Supplemental Table 1) who had no history of reproductive problems, i.e. cryptorchidism, testicular torsion, orchitis, varicocele, inguinal hernia, STD, caused pregnancy, and experienced fertility problem.

Cumulated, 2.2% of men had a sperm concentration below 5 million/mL, 4.9% below 10 million/mL, 9.0% below 15 million/mL and 31.9% below 40 million/mL. For morphology 5.7% of men had normal spermatozoa below 5%. Figure 1 summarizes the distribution of

the sperm concentration, total sperm counts, percentages and total numbers of morphologically normal spermatozoa.

Figure 1 summarises the semen results of the young men in comparison to that of the 792 fertile men. The semen variables differed between these groups, with fertile men having higher semen volume (p<0.002), sperm concentration (p<0.0001), total sperm count (p<0.0001), total number of morphologically normal spermatozoa (p<0.0001) ~~than young men, while~~and the percentage of normal spermatozoa ~~did not differ between the groups~~ (p=0.05) than young men.

The semen variables of men whose mothers had smoked during pregnancy did not significantly differ from non-exposed men, but the number of smoking mother was very small. The men's own smoking or drinking habits (not shown in Table), previous experience of cryptorchidism, testicular torsion, orchitis, sexually transmitted diseases (chlamydia, gonorrhoea or epididymitis) or previous fertility experience did not affect their semen variables.

Reproductive hormone levels differed between the groups of young men (Table 3). Men from Kawasaki and Osaka had slightly higher FSH (p<0.0001-0.049) and inhibin-B (p<0.003-0.01) levels than men from Kanazawa and Nagasaki. A similar pattern was seen for total testosterone (all p<0.0001), SHBG (all p<0.0001) and cFT (all p<0.0001). The number of men with high (mean+2SD) gonadotropin levels was very small (1-4 men for FSH 0.3-1.3%, and 2-14 for LH 0.7-4.7%). T/LH ratio of men from Kawasaki, Osaka, Kanazawa, and Nagawaki were 9.74±4.26, 8.75±3.54, 8.36±3.76, and 7.45±3.51, respectively. All pair-wise comparisons between centres except Osaka vs. Nagasaki were statistically significant (p<0.0001-0.01).

For the entire group of men the median (5-95 percentiles) sizes of the left and right testes were both 22 ml (14-28 ml). The average testis size (mean of left and right) differed significantly between the study groups, ranging from 19 to 23 ml in medians (Table 1). Overall 98.8% of study subjects had a pubic hair distribution of Tanner stage 4 or higher: Kawasaki 98.2%, Osaka 98.0%, Kanazawa 100% and Nagasaki 99.7%.

During the physical examination 27.1% of men was diagnosed with a varicocele; 14.3% stage 1, 9.1% stage 2, and 3.7% stage 3. Varicocele was on the left side only in ~~26.5% and on the right in 3.5% of men. In 9 men (0.6 % of the entire study population) the right varicocele was not concomitant with a left one~~23.6%, both sides in 2.9% and only on the right side in 0.6% of men. The presence of a varicocele was non-significantly associated with an 8% (95% confidence interval –20%;+6%) reduction in sperm concentration and 11% decline (-23%;+4%) in total sperm count. No tendencies could be detected for sperm motility or morphology. Inhibin-B and total testosterone tended to be lower in men having a varicocele, however, non-significantly (p>0.05).

DISCUSSION

Sperm concentration, total sperm count, percentage of morphologically normal spermatozoa and percentage of motile spermatozoa varied tremendously between individual participants. However, only small but statistically significant differences were detected for both semen and reproductive hormone levels between young men from the four provinces in Japan. Thus, from a biological point of view these groups of men can be regarded as similar. ~~The most important finding is that~~As expected, semen quality of the young men was significantly poorer than that of partners of pregnant women.

To our knowledge, this is the first large-scale prospectively designed study to explore potential differences in testicular function parameters in Japanese men that were not selected by any fertility status. The men were enrolled by the same type of advertisement. They were all university students. We tested for effects of various covariates and accounted for these when necessary. Thus, the comparison between the four groups is valid, and the results may serve as a reference for future studies on time trends in semen quality.

In Japan, there is not a compulsory medical examination of young men as in the Northern-European countries,[4 7] which makes it difficult to recruit men that are representative for the general population. Itoh *et al* [10] detected a median sperm concentration of 81 million/mL in 207 young men (18-22 years old) examined in 1998 in Sapporo but also con-

cluded that selection bias in the recruitment and variable ejaculation abstinence might have affected the results. In the design of our study, we therefore decided to restrict the invitation to university students to get a well-characterized study population. Men who have experienced fertility problems may be more likely to volunteer for semen studies than men without any problems.[14] Since the sperm count cannot be known without laboratory analysis, it is unlikely that any of the volunteers would have had such information. Testicular size might hint to fertility problem if it were very small. However, there was only small difference (slightly larger in the present cohort) between the testicular size of the young and the fertile men[15] in Japan. Moreover, semen variables of the healthy subgroup of young men (n=1,307) who had no history of reproductive problems were very similar to those of the entire study population (n=1,559). Likewise, it should be noted that the incidence of cryptorchidism was considerably high, which could be an indicator of recruitment bias. In the present study, however, it is unlikely to be a recruitment bias, because the information about cryptorchidism was based on the questionnaire data, i.e. history of cryptorchidism, not current cryptorchidism. This included cryptorchidism at any time, i.e. both congenital and acquired, which may have different causes and consequences. In fact, there were no differences in semen quality between the healthy subgroup of young men (Supplemental Table 1) and the entire study population (Table 2) including the men with history of cryptorchidism or other reproductive problems. We restricted the age of participants to 18-24 years assuming that the majority in such a group would not yet have any direct knowledge about their own fertility chances, since the average Japanese man fathers his first child at the age of 31.8 years.[15,16] Less than 1% of the participants had experienced fertility problems, and such problems are therefore unlikely to severely bias the obtained results. Poorly educated men may be more likely to refuse participation in studies that require delivery of semen samples.[16,17] However, higher educational level has been positively associated with semen quality.[16,17,18] Thus, the selection of university students may bias the results towards a higher level. Nevertheless, semen variables of the healthy subgroup of young men (Supplemental Table 1) who had no history of reproductive problems were very similar to those

of the entire study population (Table 2). We thus considered that semen results of the present study population was unlikely to have been subjected to a significant bias.

There were small differences in inhibin-B levels that were lower in men from Kanazawa and Nagasaki than in men from Kawasaki and Osaka. Similar trends were detected for total testosterone, SHBG, and cFT. The slightly higher inhibin-B levels in men from Kawasaki and Osaka could theoretically indicate a higher spermatogenic activity in these, which however does not fit with the sperm count findings. Also, we hesitate to draw such a conclusion since the detected inhibin-B concentrations at this level do not correlate strongly with sperm counts.[18,19] We have no explanation for the higher total testosterone in men from Kawasaki and Osaka compared to other centres. It is important to keep in mind that the adjusted hormone levels in Table 3 were adjusted for the effect of BMI, which thus does not explain the findings. T/LH ratio may inform about Leydig cell function, and therefore we also analysed it. Variation between the study centres was small but statistically significant; however, biological significance of variation in this scale is questionable.

Diagnostic accuracy of varicocele is very dependent on the clinical experience of the investigator. The high frequency of varicocele may reflect this, since the majority of the cases were grade 1. We saw no significant negative effect on semen quality, which however, is likely due to the low grade in most varicoceles.

In the European studies semen volume was assessed by weighing which now is the "gold standard", because aspiration – as used in our study – underestimates the volume by approximately 0.4 mL.[19,20,21] Thus, the true median semen volume might have been 3.4 mL, which is closer to the volumes reported from Finland, Denmark, Germany, and Spain,[3,7,8] and thus the adjusted median total sperm count in our study would have been 200 million rather than 177. Inter-observer variation may explain the higher semen volume detected in men from Kanazawa compared to other centres, because volume measurements were not under external quality control.

Three Chinese studies have described semen qualities in apparently normal men. Healthy men, 20-60 years of age, had a median sperm concentration and total sperm count

of approximately 65 million/mL and 154 million, respectively.[22,41] The youngest age group, 20-25 years old, comprised only 6.1% of the study population, and overall 83% had previously fathered a child. Another study investigated 20-40 years old men but excluded those with known andrological diseases and reported a median sperm concentration and total sperm count of 78 million/mL and 168 million.[23,32] Junqing *et al*[24,43] detected a mean sperm concentration and total sperm count of 55 million/mL and 124 million, respectively, in 22-30 years old men that underwent a premarital physical examination. Thus, the latter may be more comparable to ours, since the men presumably had little knowledge of their fertility potential. These Chinese reference values are clearly lower than the current Japanese figures, suggesting that there are regional differences in semen quality among Asian men. Interestingly, Chinese men who had at least a college education had lower sperm counts than men with a lower educational status.[24,43] If this were true also for Japanese men, the difference in semen quality between Japanese and Chinese men would be even greater than what is evident from the present results.

Other studies have shown an adverse effect of maternal smoking during pregnancy on the son's semen quality.[25,26,27,76] However, we did not find such an effect, probably because the number of smoking mothers was very small. By contrast, the smoking rates of the young men themselves looks exceedingly high for a developed nation, but this is a common trend in Japanese male, which is consistent with the figures from National Health and Nutrition Survey (47.4% in total and 60.8% in 20-29 age group in 2000)[28], as well as the results from the previous our study of the fertile Japanese men (52.8% in total).[15]

The Japanese men appeared to have higher sperm counts than men from the Northern Europe[4,5,7,27,30,29-32] but slightly lower than men from Spain.[8] Thus, Japanese men may be ranked as having better sperm counts than many populations of European young men. Nevertheless, this does not imply that impaired semen quality is not a problem among young Japanese men. Nearly 10% had sperm counts below the current WHO reference levels of 15 million/mL or 39 million[32,30] rendering them for high risk of reduced fertility in the future. Nearly one third of men had sperm concentration less than 40 million/mL, indicating

reduced fecundity.[32-35,34-37] We are therefore concerned that a significant proportion of the men may experience problems when they reach the ages when they want to reproduce.[32-35,34-37]

Our prospectively designed study showed no overall difference between men from the four investigation sites, and the semen results were similar to those previously reported for Finnish men that until now have been considered to have the best semen quality among young men from the Northern Europe. The study cohorts were, however, not completely comparable. The recently detected adverse trends in semen quality and testis cancer incidences among men from Finland suggest underlying environmental causes.[3,20] Whether the semen quality of Japanese men has changed over the years cannot be answered by our current study, but a large proportion of investigated men were shown to have sub-optimal sperm counts. These results will serve as a reference for future studies on time trends in semen quality in Japan and for comparison with future studies of university students in other countries.

Table 1: Physical appearance and self-reported information of young men from four cities in Japan.

	Entire study population (n=1559)		Kawasaki (n=658)		Osaka (n=300)		Kanazawa (n=300)		Nagasaki (n=301)		
	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	Mean (SD)	Median (5-95)	p-values
Height (cms)	172 (6)	172 (163-181)	172 (6)	172 (163-182)	172 (6)	172 (163-181)	173 (6)	173 (163-181)	172 (5)	171 (163-180)	0.01 [A]
Weight (kgs)	64 (9)	63 (52-79)	64 (11)	63 (52-80)	63 (8)	63 (52-77)	64 (8)	63 (52-80)	63 (9)	62 (52-82)	0.4 [A]
BMI (kg/m2)	21.4 (2.7)	21.0 (18.2-26.0)	21.5 (3.0)	21.0 (18.2-26.0)	21.2 (2.2)	20.9 (18.2-25.4)	21.4 (2.4)	21.1 (18.4-25.8)	21.6 (2.6)	21.3 (18.0-26.2)	0.2 [A]
Mean of left and right size (ml) [a]	21 (4)	22 (14-28)	22 (4)	22 (15-28)	22 (5)	23 (14-29)	21 (4)	21 (14-29)	20 (4)	19 (13-26)	<0.0001 [A]
Age (years) [b]	21.3 (1.6)	21.1 (18.9-24.1)	20.8 (1.4)	20.7 (18.7-23.4)	21.7 (1.6)	21.6 (19.3-24.4)	21.8 (1.6)	21.9 (18.9-24.2)	21.3 (1.7)	21.2 (18.9-24.2)	<0.0001 [A]
School education (years) [c]	15 (2)	15 (12-18)	14 (2)	14 (12-17)	15 (2)	15 (12-18)	15 (2)	15 (12-18)	15 (2)	15 (12-18)	<0.0001 [A]
Ejaculation abstinence (hours) [d]	78 (36)	65 (50-136)	76 (32)	64 (49-136)	77 (35)	64 (50-136)	82(51)	67 (51-138)	78 (28)	66 (51-136)	0.0002 [A]
		Frequency (%)		Frequency (%)		Frequency (%)		Frequency (%)		Frequency (%)	
Have (had)											
Cryptorchidism [e]		8.2		6.9		8.3		12.7		6.3	0.02 [B]
Testicular torsion		0.1		0.2		0.0		0.3		0.0	0.5 [B]
Orchitis		1.4		1.6		1.7		1.0		1.0	0.8 [B]
Varicocele		0.1		0,0		0.3		0.3		0.0	0.2 [B]
Inguinal hernia		1.1		0.7		2.3		1.0		1.0	0.2 [B]
STD [f]		2.1		2.8		1.0		1.3		2.3	0.3 [B]
Thyroid disease or diabetes		0.1		0.2		0.3		0,0		0.0	0.7 [B]
Taken medicine [g]		10.4		13.7		8.0		8.7		7.3	0.0006 [B]
Caused pregnancy		3.8		4.4		2.7		3.3		4.0	0.6 [B]
Experienced fertility problem [h]		0.9		1.8		0.3		0.0		0.7	0.03 [B]
Varicocele diagnosed in study [i]		27.1		26.8		24.3		20.0		37.5	<0.0001 [B]
Tobacco smoker		49.6		62.4		34.6		42.8		45.0	<0.0001 [B]
Exposed to tobacco in utero [j]		2,0		2.5		1.3		1.3		2.3	0.6 [B]

SD: Standard deviation.
(5-95): 5-95th percentile.
a: Size assessed by palpation. Two men had non-palpable left testicles, one from Kawasaki and one from Kanazawa due to previous orchidectomy because of testiuclar torsion.
b: Age calculated as difference between day of attendance in study and selfreported day of birth.
c: All participants were university students.
d: Ejaculation abstinence period calculated as difference between time of current ejaculation and self-reported time of previous ejaculation.
e: Not born with both testicles in scrotum (irrespective of spontaneous descend or treatment).
f: Diagnosed with epididymitis, chlamydia or gonnorhoea.
g: Taken any medication recent 3 months prior to participation in study. For 93%, 61%, 61% and 60% from Kawasaki, Osaka, Kanazawa and Nagasaki, respectively it was against either infection, allergy or pain.
h: Have had unprotected intercourse without causing a pregnancy during a 12 months period.
i: Varicocele diagnosed during this study, irrespective of previous self-reported information.
j: In utero exposed to maternal tobacco smoking.

A: Kruskal-Wallis test
B: Fisher's exact test

Table 2: Semen quality of young men from four cities in Japan.

	Observed		Adjusted
	Mean (SD)	Median (5-95)	Median (95%CI)
Semen volume (ml)			
Entire study population	2.9 (1.4)	2.7 (1.0-5.5)	3.0 (2.8-3.2)
Kawasaki	2.7 (1.3)	2.5 (0.9-5.2)	2.8 (2.7-3.2)
Osaka	2.9 (1.4)	2.7 (1.0-5.6)	2.9 (2.7-3.2)
Kanazawa	3.3 (1.5)	3.0 (1.2-6.4)	3.3 (3.1-3.6)
Nagasaki	2.9 (1.4)	2.8 (0.9-5.4)	3.0 (2.8-3.2)
p-value			p=0.0006
Sperm concentration (mill/ml)			
Entire study population	73 (58)	59 (10-185)	59 (52-68)
Kawasaki	71 (61)	55 (9-185)	57 (48-66)
Osaka	75 (61)	60 (9-195)	61 (50-74)
Kanazawa	72 (55)	60 (7-183)	61 (51-73)
Nagasaki	76 (54)	64 (12-181)	61 (51-74)
p-value			p=0.138
Total sperm count (mill)			
Entire study population	201 (183)	159 (18-509)	177 (153-206)
Kawasaki	185 (193)	143 (17-472)	161 (135-191)
Osaka	202 (178)	163 (28-508)	179 (146-221)
Kanazawa	228 (184)	185 (15-546)	201 (165-246)
Nagasaki	201 (161)	166 (22-531)	183 (150-224)
p-value			p=0.002
Motile spermatozoa (%)			
Entire study population	67 (14)	69 (42-88)	67 (65-69)
Kawasaki	66 (14)	68 (40-87)	65 (62-67)
Osaka	67 (12)	67 (52-84)	64 (61-67)
Kanazawa	62 (14)	64 (38-82)	60 (57-63)
Nagasaki	76 (14)	78 (48-93)	75 (73-78)
p-value			p<0.0001
Morphologically normal spermatozoa (%) [a]			
Entire study population	10.3 (6.0)	9 (2.5-21.5)	9.9 (8.8-10.9)
Kawasaki	10.5 (6.2)	9 (2.5-23)	9.3 (7.9-10.6)
Osaka	9.2 (5.2)	8.5 (2.5-19)	8.5 (7.2-9.8)
Kanazawa	10.0 (6.1)	8.8 (2-21)	9.3 (8.0-10.5)
Nagasaki	11.7 (6.1)	11 (3-23)	11.2 (10.0-12.4)
p-value			p<0.0001

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis.
Semen volume, sperm concentration and total sperm counts adjusted to a period of ejaculation abstinence of 96 h for a 21 years-old-man
Motility and morphology adjusted for a 21 years-old-man, winter season
See text for further explanation.
p-value: Based on regression analyses of cubic root transformed values, comparing all four groups.
a: Morphology results only available for 869 men.

Table 3: Reproductive hormone levels of young men from four cities in Japan

	Observed		Adjusted
	Mean (SD)	Median (5-95)	Median (95%CI)
FSH (U/l)			
Entire study population	2.5 (1.3)	2.2 (1.1-4.9)	2.3 (2.2-2.5)
Kawasaki	2.7 (1.3)	2.4 (1.1-5.2)	2.4 (2.3-2.6)
Osaka	2.5 (1.3)	2.2 (1.1-5.0)	2.3 (2.1-2.4)
Kanazawa	2.3 (1.3)	2.1 (1-4.4)	2.1 (1.9-2.3)
Nagasaki	2.3 (1.2)	2.1 (0.9-4.4)	2.1 (1.9-2.2)
p-value			p<0.0001
Inhibin-B (pg/ml)			
Entire study population	202 (64)	197 (110-314)	190 (181-199)
Kawasaki	207 (64)	202 (113-322)	191 (181-201)
Osaka	214 (67)	210 (117-336)	193 (182-205)
Kanazawa	191 (62)	190 (104-304)	177 (164-191)
Nagasaki	188 (60)	182 (103-286)	176 (165-187)
p-value			p=0.003
LH (U/l)			
Entire study population	3.2 (1.3)	2.9 (1.5-5.6)	2.7 (2.6-2.9)
Kawasaki	3 (1.3)	2.8 (1.4-5.3)	2.7 (2.5-2.8)
Osaka	3.5 (1.4)	3.2 (1.8-6.4)	3.0 (2.9-3.2)
Kanazawa	3.3 (1.4)	3.1 (1.6-5.8)	2.8 (2.6-3.1)
Nagasaki	2.9 (1.0)	2.8 (1.5-4.9)	2.6 (2.4-2.8)
p-value			p<0.0001
Testosterone (nmol/l)			
Entire study population	25 (8)	24 (14-39)	26 (25-27)
Kawasaki	26 (7)	25 (15-39)	26 (25-27)
Osaka	27 (9)	26 (15-42)	27 (26-28)
Kanazawa	22 (7)	21 (12-35)	23 (21-24)
Nagasaki	22 (7)	21 (13-36)	23 (21-24)
p-value			p<0.0001
SHBG (nmol/l)			
Entire study population	28 (11)	26 (13-46)	27 (26-28)
Kawasaki	30 (11)	29 (14-49)	28 (27-30)
Osaka	29 (11)	27 (14-50)	26 (25-28)
Kanazawa	24 (9)	23 (12-41)	23 (21-24)
Nagasaki	24 (9)	23 (12-42)	23 (21-24)
p-value			p<0.0001
cFT (pmol/l) [a]			
Entire study population	607 (192)	585 (326-949)	638 (613-663)
Kawasaki	619 (181)	605 (339-924)	630 (604-657)
Osaka	672 (214)	649 (371-1029)	675 (642-710)
Kanazawa	556 (183)	527 (298-894)	589 (552-628)
Nagasaki	566 (177)	550 (316-922)	583 (552-615)
p-value			p<0.0001

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis, adjusted to blood sampling at 10:00 am in winter season, representing af 21 years-old man having a BMI of 21.
p-value: Based on regression analyses of natural logarithmic transformed values, comparing all four groups.
a: Calculated free-testosterone.

Ethics

This study got the approval of the Ethics Review Board in each university and hospital. All participants gave their written consent before participating in the study.

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Data sharing statement

There is no additional data available.

Contributor statement

NJ, NES, JT, SN, MNM, MY and TI conceived and designed this study.

Acquisition of data: TI, MY, SN, KY, KB, MN, EK, JK, AO, KM, AT, HK, and JE.

Data analysis was performed by MNM, NJ and TI.

NJ, TI, SN and JT drafted the manuscript.

MV assessed the sperm morphology smears.

JK, EK, MN, KB, KY, AT, KM, AO, JE, HK and TI performed the physical examinations and collected the data.

All authors participated in the interpretation of data.

- Revising the article critically for important intellectual content: All authors.
- Final approval of the version to be published: All authors.

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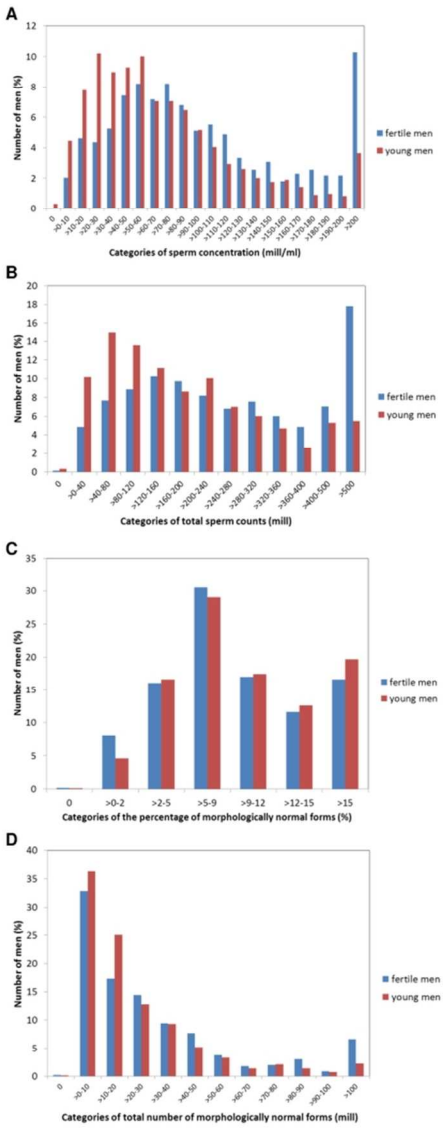

The semen results of the 1,559 young men in comparison with those of the 792 fertile men. The semen variables differed between these groups, with fertile men having higher sperm concentration (A, p<0.0001), total sperm count (B, p<0.0001), total number of morphologically normal spermatozoa (D, p<0.0001) than young men, while the percentage of normal spermatozoa did not differ between the groups (C, p=0.05).
90x127mm (300 x 300 DPI)

Table A1: Semen quality of subgroup of 1,307 healthy young men from four cities in Japan.

	Observed		Adjusted
	Mean (SD)	**Median (5-95)**	**Median (95%CI)**
Semen volume (ml)			
Entire study population	2.9 (1.4)	2.8 (1-5.6)	3.0 (2.8-3.1)
Kawasaki	2.8 (1.3)	2.6 (0.9-5.3)	2.8 (2.6-3.0)
Osaka	2.9 (1.4)	2.7 (1-5.6)	2.9 (2.6-3.1)
Kanazawa	3.3 (1.5)	3 (1.2-6.5)	3.2 (2.9-3.5)
Nagasaki	3 (1.4)	3 (1-5.4)	3.0 (2.8-3.3)
p-value			p=0.011
Sperm concentration (mill/ml)			
Entire study population	74.4 (59.8)	60.2 (10.7-187)	62 (54-70)
Kawasaki	72.5 (62.6)	56.8 (10.6-187.5)	58 (50-68)
Osaka	74.7 (62.2)	59.1 (10.5-195.3)	65 (55-78)
Kanazawa	73.5 (56.1)	61.3 (7.5-183.3)	61 (51-72)
Nagasaki	78.9 (54.7)	71.3 (12-181.4)	66 (55-78)
p-value			p=0.121
Total sperm count (mill)			
Entire study population	206.3 (189.8)	165.3 (19.7-514.5)	181 (158-209)
Kawasaki	192.5 (203.5)	150.1 (17-494.7)	164 (140-194)
Osaka	198.2 (178.8)	159.8 (28.4-496.4)	188 (154-229)
Kanazawa	232.1 (192.2)	185.4 (17-561.3)	192 (158-234)
Nagasaki	219.3 (163.1)	183.8 (31.2-553.2)	198 (164-239)
p-value			p=0.008
Motile spermatozoa (%)			
Entire study population	67.4 (14.4)	69 (42-88)	66 (64-68)
Kawasaki	66.1 (14.4)	67 (40-86)	64 (62-67)
Osaka	66.8 (11.8)	67.5 (52-84)	64 (61-67)
Kanazawa	62.4 (13.7)	64 (38-83)	60 (57-63)
Nagasaki	75.6 (14.3)	78 (49-92)	75 (72-77)
p-value			p<0.0001
Morphologically normal spermatozoa (%) a			
Entire study population	10.4 (6.1)	9.5 (2.5-22)	9.7 (8.8-10.5)
Kawasaki	10.7 (6.4)	9 (2.5-23)	9.4 (8.1-10.8)
Osaka	9.2 (5.3)	8.5 (1.5-19)	8.3 (7.1-9.5)
Kanazawa	10 (6.2)	8.5 (1.5-21.5)	9.1 (8.0-10.2)
Nagasaki	11.8 (6)	11 (3.5-22.5)	11.1 (10.0-12.2)
p-value			p=0.0007

Observed: Results based on raw data.
SD: Standard deviation.
(5-95): 5-95th percentile.
Adjusted median and 95%CI (confidence interval) calculated by linear regression analysis.
Semen volume, sperm concentration and total sperm counts adjusted to a period of ejaclation abstinence of 96 h for a 21 years-old-man.
Motility and morphology adjusted for a 21 years-old-man, w inter season.
See text for further explanation.
p-value: Based on regression analyses of cubic root transformed values, comparing all four groups.
a: Morphology results only available for 727 men.

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cross-sectional studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 4
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3, 4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-5
Objectives	3	State specific objectives, including any prespecified hypotheses	4, 5
Methods			
Study design	4	Present key elements of study design early in the paper	4, 5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5, 6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	5, 6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	3, 7, 8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5-8
Bias	9	Describe any efforts to address potential sources of bias	8, 9
Study size	10	Explain how the study size was arrived at	5-6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7-10
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-10
		(*b*) Describe any methods used to examine subgroups and interactions	8, 9
		(*c*) Explain how missing data were addressed	N/A
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	5, 6
		(*e*) Describe any sensitivity analyses	7
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5, 6
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	6, 17 (Table 1)
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers of outcome events or summary measures	10-12, 18, 19 (Table 2 and 3)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10-12, 18, 19 (Table 2 and 3)
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	3, 4, 12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-16
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14-16
Generalisability	21	Discuss the generalisability (external validity) of the study results	15, 16
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	20

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.