Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

Bias and confounding in studies of sperm counts

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Abstract: Impairment of semen quality has become a major topic in the public health debate the last 5 years. Some studies have shown a decline of sperm concentration during the past decades. Other studies, however, did not show a deterioration of sperm quality. All studies are characterised by the lack of comparable data on semen sample collection and on methodology used for semen analysis. Even studies performed in one centre do not guarantee the use of the same technique for sperm counting over years. It has also been well established that abstinence time, life style, drugs, smoking, alcohol abuse, stress and increase of scrotal temperature during fever and due to the presence of a varicocele may decrease sperm concentration. None of the studies have examined the potential impact of occupational conditions on reproduction. Nevertheless, certain professions are associated with reduced sperm quality. Furthermore, regional and ethnic differences may contribute to differences of sperm quality. Therefore, no definite answer can be given whether there is a time-related decline in sperm concentration.

INTRODUCTION

The possible decline in human semen quality has become a major issue of concern the last few years. Decline in sperm count and/or sperm concentration, decrease of sperm motility and an increase of morphological abnormal cells have been described as major factors to define impairment of semen quality. There is good evidence for positive associations between semen characteristics and the likelihood of achieving a pregnancy. However, the possible decline in semen quality has not yet resulted in reports about a reduction in male fertility (ref. 1). Moreover, the outcome of the different studies is not unequivocally a sperm decrease (Table 1, refs 2–16). This makes it even more difficult to find a cause effect relationship between decreasing sperm quality and the hypothesised exposure of pregnant women and their male offspring to hormone disrupters, and especially environmental oestrogens. The most commonly used parameter in the various publications are sperm concentration (= sperm density, millions/mL), and total sperm count per ejaculate (millions/ejaculate). In this paper we will only discuss factors affecting sperm counting, since we believe that in many studies the assessment of sperm motility and morphology was poor and/or difficult to evaluate. For instance the assessment of sperm morphology has changed over the years. In 1941 Hotchkiss reported that 88% of spermatozoa had normal morphology (ref. 17); this figure must be based upon another definition of normal morphology, as is used today. Fifteen years ago, the morphology of a semen sample was considered to be abnormal if more than 50% of the spermatozoa had abnormal morphology. Today, more than 70% of the spermatozoa has to be abnormal for the indication 'teratozoospermia' (abnormal morphology), but even this value has been argued to be too low. Some laboratories consider samples with 86% abnormal spermatozoa still normal (ref. 18). The cause for the rapid lowering of the cut-off value normal-abnormal is not the deterioration of sperm quality, but the way of evaluating sperm morphology. The decrease of the percentage normal

sperm is caused both by the use of stricter morphology criteria, as proposed by Eliasson (ref. 19) and Kruger *et al.* (ref. 17), and a more critical attitude of technicians due to their growing experience. Moreover, even the World Health Organisation (WHO) manual does not give an unequivocal description of normal morphology (ref. 20).

Table 1. Summary of publications on sperm concentration since 1992

Author	Study design and period	Number of men included	Abstinence days	Methodology Sperm assessment	Results Mean (median)
ref. 2	Meta-analysis 1940–1990	14 947 men with known and unknown fertility	n.m.*	n.m.*	Decline $113 \rightarrow 66 \times 10^6$/mL
ref. 3	Retrospective 1973–1992	1351 donors (fathers)	3–5 advised	WHO'80	Decline $89 \rightarrow 60 \times 10^6$/mL
ref. 4	Retrospective 1977–1993	3285 male partners of infertile couples	3–5 advised	Neubauer	Decline $51 \rightarrow 39 \times 10^6$/mL
ref. 5	Retrospective 1984–1995	577 donors and students born between 1951–1973	3–4 advised	WHO '80, '87, and '92	Decline $98 \rightarrow 78 \times 10^6$/mL
ref. 6	Retrospective 1970–1990	4518 men	n.m.*	n.m.*	Decline $72 \rightarrow 65 \times 10^6$/mL
ref. 7	Retrospective 1989–1994	7714 men with normal sperm before IVF	2–4 advised	n.m.	Decline $94 \rightarrow 88 \times 10^6$/mL
ref. 8	6 Finnish studies 1958–1992	849 fertile and men with unknown fertility	n.m.*	n.m.*	No decline $111 \rightarrow 124 \times 10^6$/mL
ref. 9	Retrospective 1978–1989	183 male partners of infertile women	n.m.	n.m.	No decline† $101 \rightarrow 95 \times 10^6$/mL
ref. 10	Retrospective 1977–1992	302 donors (fathers)	n.m.*	n.m.*	No decline 83×10^6/mL
ref. 11	Retrospective 1977–1995	416 donors and students	3–5 advised	WHO '87	No decline $67 \rightarrow 64 \times 10^6$/mL
ref. 12	Retrospective 1967–1994	5,253 infertile men	3–5 advised	Bürker Bürker-Türk	No decline $134 (94) \times 10^6$/mL
ref. 13	Retrospective 1980–1995	689 donors and men participating in studies	≥2 advised	WHO '80, '87, and '92	No decline $(69) \times 10^6$/mL
ref. 14	Retrospective 1985–1995	718 male partners of infertile couples	>3 advised	Makler	Increase $46 \rightarrow 64 \times 10^6$/mL
ref. 15	Retrospective 1970–1994	1283 pre-vasectomy 64% proven fertility	3–10 advised	Neubauer Makler	Increase $77 \rightarrow 89 \times 10^6$/mL
ref. 16	Retrospective 1972–1993	510 fertile men participating in studies	2–7 advised	Coulter counter	Increase $46 \rightarrow 52 \times 10^6$/mL

* Not mentioned.

† Decline ($105 \rightarrow 76 \times 10^6$/mL) in couples living in the Thames Water supply Area.

It is well documented that the variability of a man's semen characteristics may be considerable (refs 21–22). This might be due to a number of factors, such as period of abstinence, recent and present disease, use of certain medication, drug- and alcohol abuse, and the circumstances under which the sample is produced. In addition to the well-known fluctuations of semen quality, also the methods of assessment have many limitations and inherent errors. These inadequacies are augmented by different methods still being employed to count sperm cells. Although the WHO has made recommendations to standardise the procedures for the analysis of human sperm (ref. 20), these have not been generally applied (ref. 23). It was shown in an external quality control program, that the semen analysis showed wide between laboratory variation in both the sperm concentration and morphology assessments (ref. 23). Part of the variation in sperm count can be explained by the use of different counting chambers, and the way of diluting the sperm samples. Moreover, there is only circumstantial evidence that quality control procedures are routinely performed in most laboratories. In summary, two major groups of

confounding factors effect sperm counts: One group concerns the conditions before and during the production of the sample, the other the laboratory methods of sperm counting.

The comparability of the publications is limited because they comprise different populations (infertile men, sperm donors with or without known fertility, and men cryopreserving sperm before vasectomy) in different countries. None of these populations can be regarded as representative for the normal population (ref. 1).

CONDITIONS BEFORE AND DURING SAMPLE PRODUCTION

Effect of abstinence period and ejaculatory frequency

It has been well established that ejaculatory abstinence and sperm concentration are positively correlated. Most conspicuous in the study by Pellestor *et al.* (ref. 24) was the increase in sperm volume and sperm count after various ejaculatory abstinence periods from 2 to 18 days. A statistically significant lower sperm concentration has been observed after 1 day of abstinence compared with 4 days of abstinence. This difference was only significant in normozoospermic samples of infertile men (ref. 25). Total sperm count decreased markedly in medical students when there was a high frequency of ejaculation (ref. 26). Sperm concentration followed the same pattern but less pronounced. Age can be a confounder for duration of abstinence. Long abstinence periods are found commonly in older men. Correct interpretation of results does require that the actual duration of abstinence is known. Obviously, a component of sample variation, on a population basis, can be attributed to variations in prior abstinence.

Method of sperm collection

It has been reported that the quality of an ejaculate produced during coitus is superior to that produced by masturbation with respect to semen volume, sperm concentration, progressive motility, and even morphology (ref. 27). This has been explained by a higher level of sexual arousal during coitus compared to masturbation. However, we could not confirm that sexual arousal is a major factor determining sperm quality (ref. 28). Semen quality is also better when collected via intercourse than obtained via coitus interruptus (ref. 29).

PHYSICO-CHEMICAL CHARACTERISTICS OF THE EJACULATE

At ejaculation, spermatozoa are expelled from the cauda epididymidis and vas deferens, mixed with secretory products of the accessory glands, and emitted to the exterior along the urethra. The first part of the ejaculate contains most of the spermatozoa suspended in predominantly prostatic fluid. The last fraction contains only residual spermatozoa from the epididymis in vesicular fluid. Spontaneous coagulation of the ejaculate occurs rapidly after ejaculation due to components of the seminal vesicles. Proteolytic enzymes from the prostate effect subsequent liquefaction. Abnormalities of liquefaction may lead to an inhomogeneous sample, which may have an affect on the number of sperm cells present in the aliquot taken for examination. Because of the viscous nature of semen, errors may also arise from diluting semen for sperm concentration with air-driven pipettes and the inhomogeneous character of some samples (ref. 30).

Effects of counting-chambers characteristics

Various counting chambers are being employed in recent years to assess sperm concentration. A major problem is the depth of the counting chambers, which, depending upon type, vary between 10 μm and 100 μm. It is exceptional if laboratories have facilities to check the depth of counting chambers. Therefore it is not surprising that both the variance within and between the various types of counting chambers have been shown to be high (ref. 31). In addition many chambers are convenient to use, but they lack the accuracy of the WHO recommended haemocytometer (ref. 20).

Technician repeatability

Technician-dependent effects and technician reading make significant contributions to the overall variance of sperm counting. The training and experience of technicians are obviously important Marked variations in the estimates of sperm concentration have been described when samples were examined repeatedly by one or more technicians, coming from different laboratories and using the same counting chamber (ref. 32). Nevertheless within one laboratory intra- and inter-technician variability in the assessment of sperm concentration can be low (ref. 33).

CONDITIONS AFFECTING TESTICULAR SPERM PRODUCTION

There are many complicating factors, that are mostly not addressed in studies on sperm quality (Table 2). However, the evidence that these confounding factors may impair sperm quality is not always substantial.

Table 2. Summary of factors that may affect sperm concentration

Methodology of sperm analysis	• Lack of standardisation of sperm collection
	• Lack of standardisation of laboratory procedures
Complicating factors	• Season of sampling
	• Lifestyle
	• Profession
	• Diseases
	• Medication
	• Stress
	• Age
Trends	• Higher prevalence of varicoceles associated with increased body height
	• Changes in lifestyle
	• Environmental changes
	• Changes in occupational activities
Fluctuations over the year	• Seasonal changes
Influence of geography	• Ethnicity
	• Fertility status
Influence of study population	• Changes in composition of the population visiting fertility (related) clinics
	• Region of living

Various illnesses may affect semen characteristics. A semen sample produced for analysis up to 10 to 12 weeks after febrile illness (= the time interval between spermatogonial division and the appearance of the resulting spermatozoa in the ejaculate) may have a low sperm concentration. Fever may even lead to azoospermia. Normal sperm concentration has been described to be lower in summer compared with winter. Also a moderate increase of the scrotal temperature may have deleterious effects on spermatogenesis, leading to severe decrease of sperm concentration.

The increase of scrotal temperature due to wearing tight underwear, taking hot baths and sauna has been associated with a decrease of sperm concentration (ref. 34). The presence of a varicocele may lead to impairment of scrotal temperature regulation. Whilst normally, cooling of the scrotum will be best when a men is walking or standing in an upright position, in men with a varicocele, scrotal temperature is increased when these men are standing (ref. 35). The prevalence of the presence of a varicocele in the general population is estimated to be between 10 and 15%. In a population of infertile men we have found a prevalence of 29% (ref. 36). Moreover, it has been shown that treatment of a varicocele will both restore scrotal temperature regulation and cause an increase of sperm concentration (refs 37–38). The finding that the prevalence of varicoceles was remarkably higher (45%) in very tall men (ref. 39), might be extremely important, since the body height of men is increasing in developed countries. Also Handelsman *et al.* observed that men with varicocele were significantly taller than men without varicocele. (ref. 40). At the same time one has to realise that an increase in body height and/or weight is

accompanied by an increase in testis size and sperm production(ref. 41–44). As both factors are operating simultaneously, it is hard to say what factor dominates.

Some therapeutic drugs such as Sulfasalazine, and cytostatic drugs are known to have direct side effects causing infertility.

Smoking (ref. 45), alcohol abuse (refs 46–47), drug abuse, and both physical and psychological stress (refs 48–49) have been observed to have a negative effect on spermatogenesis. Particularly well studied are the negative effects of physical stress, especially endurance training, on sperm quality (refs 50–53). Acute stress resulting from an earthquake reduced sperm concentration and motility (ref. 54).

Reduction of sperm quality has been found in relation to certain professions (agricultural workers using pesticides, welders, workers exposed to radiation, ethylenebromide, glycolethers, and lead). However, the majority of chemical agents to which men might be exposed have not been evaluated with respect to an effect on reproductive functions. Other factors that might have a negative effect on sperm concentration are cryptorchidism, and sexually transmitted diseases and the use of anabolic steroids.

REGIONAL DIFFERENCES AND COMPOSITION OF THE STUDY POPULATION

Sperm count may vary among different geographic locations. Variations in sperm counts have been described between different states in the USA, with values from New-York being highest (ref. 15). Fish *et al.* (ref. 55) analysed 20 of the 61 studies, cited in the Carlsen meta-analysis (ref. 2), and all comprising 100 or more participants. They gave special emphasis on geographical distribution in relation to the time period. Eventually, they observed that all five studies published before 1970 were performed in New York, where sperm counts are reported to be higher than not only in other parts of the USA but also in other parts of the world. Twelve of the 15 studies published after 1970 also included third world countries, where sperm counts might be low.

Becker *et al.* (ref. 56) and Swan *et al.* (ref. 57) also arranged the 61 studies, that were included in the Carlsen paper (ref. 2), according to geographical regions. Both authors concluded that in the USA a decline of sperm concentration could be observed between 1940 and 1990. Swan was also able to find a significant decrease of the mean sperm counts in Europe between 1971 and 1990, which could not be confirmed by Becker. Swan *et al.* in their analysis controlled for potential confounding factors such as abstinence time, age, specimen collection method, goal of the study and geographical location.

The mean sperm concentrations in the New York region, presented in 4 studies published between 1938 and 1951, range from 110.7 to 134.0 x 10^6/mL. Interestingly, the recent study by Fisch *et al.* (ref. 55) showed a mean sperm concentration in New York of 131.5 x 10^6/mL in the period between 1970 and 1994., which is not in favour for a decline of sperm concentration.

Regional differences have also been shown between 8 French CECOS (Centres d' Etude et de Conservation des Oeufs et du Sperme humains) centres, including Paris (ref. 3) and Toulouse (ref. 10) in the period 1973–1993 (ref. 58). The mean values range from 82 to 10^2 x 10^6/mL., values being lowest at Grenoble and highest at Caen.

A few examples of other complicating factors in the large studies, cited in the Carlsen paper (ref. 2) are shown in Table 3. They refer to differences in the composition of the study groups. Also, in all these studies, there is no adequate description of the methodology of sperm assessment.

Differences in ethnic composition can also have effects on sperm concentration. Values for sperm concentration in Thai males are lower than in Caucasian males (ref. 42). The mean testicular size in Chinese men is smaller than that reported in Caucasians, but sperm count is comparable (ref. 41).

Table 3 Example of complicating factors in four large studies, cited in the Carlsen meta-analysis

Study	Number of men included	Background	Sperm collection	Concentration $(x\ 10^6/mL)$
ref. 57	200 husbands of pregnant women	Indigent patients, and patients of a pay clinic	33 condom 167 coitus interruptus	121
ref. 58	100 men (98 unmarried)	Majority medical students	Masturbation in glass containers	134
ref. 59	100 fertile husbands of pregnant women	Already 2 or more children	Number of cases coitus interruptus	101
ref. 60	1000 fertile husbands of pregnant women	Majority Italian birth or extraction	Masturbation in glass containers	107

CONCLUSION

The meta-analysis by Carlsen and co-workers (ref. 2) published in 1992 revealed a significant decline of sperm concentration (113 to 66 x 10^6/mL) over 50 years between 1940 and 1990, based on 61 publications. It is evident that major confounding factors and bias may determine the results of a study. If these factors are not mentioned for the studied population, comparison of studies is difficult and conclusions may be wrong.

Both a decline and absence of decline of sperm quality have been described. The majority of publications have appeared since the aforementioned meta-analysis (Table 1). All these studies have made the observations over time in one laboratory and have used an approximately comparable population. Analysis of the various studies that showed evidence for a decline of sperm concentration (refs 3–7) did not take all the different methodological effects into account, which might have invalidated the conclusions. Similar methodological flaws are present in the studies that did not report a decline of sperm concentration (refs 8–16). Recruitment methods or selection criteria are poorly described in most publications. Confounders like age, life style, illnesses, stress, occupation, eating habits, presence of varicoceles and exact abstinence time have not been mentioned in most studies. Most conspicuous is the absence of standardisation of sperm parameter assessment in most studies. Comparison of the studies is also hampered by the possibility of geographical differences, fluctuation of sperm counts over the years and differences in the studied populations, e.g. infertile men, sperm donors, before vasectomy men.

The meta-analysis of 61 studies published by Carlsen *et al.* created much debate. Since this meta-analysis a number of re-analyses appeared, using alternative statistical procedures. Olson *et al.* (ref. 63) came to the same conclusion that the mean sperm concentration decreased from 1940 to 1990.

However, the question is still unanswered if data on sperm quality may be analysed and compared in view of the numerous complicating factors that contribute sperm quality. It has been questioned whether the decline in sperm quality might be explained by environmental factors. Indeed, environmental exposure to endocrine disrupters increased over the last decades, but it is actually impossible to conclude that a decline of sperm concentration, if any, can be attributed to environmental factors, especially oestrogens.

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