

Age Dependence of Murine Spermatogenesis

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The dependence of spermatogenesis function on murine age shall be investigated. Thus, testicular samples of at least 10 NMRI mice per group aged 0 to 26 months are analysed by flow cytometry after staining the DNA with DAPI. The aim of this study is to be able to account for the influence of age on mice.

There are no changes in spermatogenesis in mice aged 11 weeks up to 16 months with respect to testis weight and to the frequency of different testicular cell types. From 16 months onwards, there is a tendency to a reduced spermatogenesis function: The frequency of round spermatids is decreased. In addition, there is an increased chromatin dispersion in elongated spermatids with age. Thus, older mice (>16 months) should not be used for experiments e.g. on radiation and drug effects any more. The frequency of abnormal diploid spermatozoa does not increase with age.

Introduction

In contrast to the somatic cells of the metazoic body, cells of the *idioplasma* (germ line) which finally become germ cells in the *haplophase* are potentially immortal. However, the influence of age of the individual on gametogenesis has long been a matter of debate. In oogenesis, the situation is more obvious: Mutational frequency increases with age increasing e.g. the frequency of Down's syndrome in man, before, at a species dependent age, ovulation and thus fertility end by hormonal regulation. As concerns spermatogenesis, it is assumed that the production of spermatozoa which is dependent on pituitary FSH (follicle stimulating hormone) and on androgens may be retained up to a high age. However, there obviously is an increase of certain human genetic disorders with paternal age that have been linked to genetic mutations such as achondroplasia, Apert, Marfan and Down's syndrome (Tunte *et al.*, 1967; Murdock *et al.*, 1972; Smith, 1972; Kovaleva 1992). Also, the ease with which conception is achieved decreases after 25 years in man (MacLeod and Gold, 1953).

Spermatogenesis is the suitable system for assessments of fertility and genetic risk in the male gonad. Spermatogenesis is a steady state system

providing large numbers of cells for analysis. In vertebrates, the formation of spermatozoa is localised in the testes. The gonocytes yield spermatogonia. The development of male germ cells in the seminiferous epithelium involves three main phases: spermatogonial multiplication, meiosis, and spermiogenesis. At first, spermatogonia undergo multiple mitotic divisions and get spermatocytes that grow and divide twice during meiosis to become round spermatids. These cells differentiate during spermiogenesis to elongated spermatids and finally leave the testis as spermatozoa.

Not only in biology, but also in medicine, there is an increasing interest in germ cell reactions, because of the general ageing of the population, and because more effective therapies e.g. with radiation or chemicals lead to long term survival of man. The reaction of spermatogenesis to radiation was proposed early as an assay in radiotherapy because of the high radiosensitivity of particular cell types (Regaud and Ferroux, 1927). The general radiosensitivity pattern of germ cells is similar in man and mouse, so that the mouse may serve as a model for man (Meistrich and Samuels, 1985). The mouse only has a life expectancy of about two years. Thus, this mammal seems well suited for studies on the age dependence of spermatogenesis function.

To be able to discriminate effects of radiation or chemicals from those of the age of mice, these

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influences are evaluated here. Changes in testis weight, the genetics, kinetics and the frequency of the different testicular cell types are analysed.

Using flow cytometry, six groups of spermatogenic cells can be differentiated: 1. haploid elongated spermatids (1 cc*), 2. haploid round spermatids (1 c), 3. diploid (2 c) cells (Leydig and Sertoli cells, epithelial cells, spermatogonia in the G₁-phase of the cell cycle, and preleptone spermatocytes), 4. cells synthesizing DNA (mainly spermatogonia and primary spermatocytes), 5. 4c-cells, mainly primary spermatocytes in pachytene, and some (G₂+M)-spermatogonia, and 6. diploid elongated spermatids which are abnormal. These polyploid germ cells arise if the second meiotic division does not take place, at least in *Saccharomyces cerevisiae* (Sora and Magni, 1986). In spite of their gross genetic abnormality, they undergo spermiogenesis. Their only morphologic feature in mice is an oversized head (cf. Hacker-Klom *et al.*, 1989). Besides diploid spermatozoa, numerical chromosome aberrations may be detected by an increase in the dispersion in the DNA histograms (Otto and Oldiges, 1980): The coefficient of variation (CV) thus is not only an indicator of the technical and optical measuring accuracy but also indicates the biological variance. Mice were investigated over their whole life span of 0 to 26 months of age.

Materials and Methods

Male NMRI mice (*Mus musculus*, originally from the Institut für Versuchstierzucht (Hannover) aged 0 to 26 months were used. These mice were bred in our own mouse colony under natural diurnal rhythm. Mice were killed by cervical dislocation. Testes of at least 10 mice were weighted. For flow cytometric analysis, the testes were minced with surgical blades after removal of the *tunica albuginea*, fat, epididymis, and connective tissue (Hacker-Klom, 1994). A particle analysis

system PAS II (Partec AG, Münster) was used for the flow cytometric analysis.

Sample preparation and staining procedure with the DNA specific fluorochrome 4',6-diamidino-2-phenylindole·2HCl (DAPI) has been described before (protocol 1, cf. Otto and Oldiges, 1986). The composition of a DNA histogram of testicular cells as measured by flow cytometry was verified by cell sorting (Tatchen *et al.*, 1989). The calculation of the different testicular cell types was performed using the cumulative frequency distribution (Meier, 1983). The coefficients of variation which allows to determine the accuracy of measurements were about 2% for peak III in each DNA histogram. Each testis was analysed separately. Single values for the testes are indicated in Figs 1 and 3–5

Results

Testis weight

Testis weight increases from birth to the 10th week by a factor of about 56 from 2.5 ± 0.5 mg to 140 ± 11 mg. The significant changes occur after the 11th week (Fig. 1).

A tendency to reduction of testis weight with increasing age is observed in mice. The correlation coefficient is (-0.75). Testis weight is only reduced in some older mice. In some mice, both testes are lighter than normal, e.g. in mouse no. 145

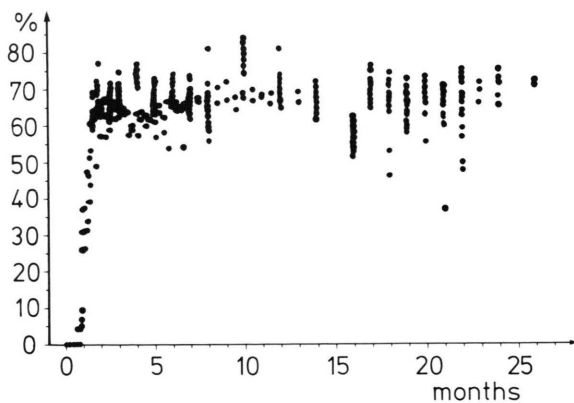


Fig. 1. Testicular weight (mg) as a function of the age of the mice. 576 single values from two separate experiments are pooled. Each value represents one testis. Single values are indicated to show that in only some older mice, deviations from normality occur. In Figs 3 to 5, the data are displayed similarly.

* "c" means "content" of DNA; a 2 c DNA content corresponds to a diploid chromosome set; "1 cc" means a haploid chromosome set, but since elongated spermatids do not stain proportionally to their DNA content because of their highly condensed chromatin, one gets the false impression that they have a lower than 1 c DNA content.

(18 months old, both testes weight only 85 mg). In other mice, one testis is normal whereas the other has a reduced weight, e.g. mouse no. 185 (21 months old), the right testis weights only 72 mg, and the left testis weights 124 mg. Because of deviations from average by some individuals, the variability becomes higher with increasing age of the mice.

DNA histograms

Representative DNA histograms (Fig. 2) are chosen to show the age dependence of murine spermatogenesis. Only 2c, S and 4c cells are found in the testes at birth (83 %, 10 %, 7 % respectively, see Fig. 2 a).

At the age of $1\frac{1}{2}$ weeks, the percentage of 4c cells increases to 12 % (peak IV, representing primary spermatocytes and ($G_2 + M$) spermatogonia, Fig. 2 b). Already there are some round spermatids in the testes (peak II). At the age of three weeks, the percentages of 4c cells (peak IV) and round spermatids (peak II) are increased to 26 % and 4 %, respectively (Fig. 2 c).

In a mouse that is $4\frac{1}{2}$ weeks old, the proportions of elongated and round spermatids have increased further (Fig. 2 d).

A mouse aged 6 weeks has nearly adult haploid germ cell frequencies of 59 % (8 % elongated and 51 % round spermatids, see Fig. 2 e).

In a mouse aged 12 weeks, there are 65 % haploid germ cells (13 % elongated and 52 % round spermatids, see Fig. 2 f). In the DNA histogram of a mouse aged 12 months, 64 % of the cells in the testis are haploid germ cells (17 % elongated and 47 % round spermatids, see Fig. 2 g). The DNA histograms of two mice that were 18 and 21 months old do not differ very much from that of the mouse aged 12 months (Fig. 2 h, i).

In a mouse aged 21 months (Fig. 2 j, mouse no. 185), haploid germ cells in the right testis are reduced to only 35 %. Testicular weight is reduced to 57 % of normal (0.0719 g); the weight of the left testis and the percentage of haploid germ cells are normal with 0.1237 g and 66 % (Fig. 2 k). In a mouse aged 22 months, the right testis also shows reduced percentage of haploid germ cells. The left testis is normal weight, and haploid germ cells are normal in number (Fig. 2 l, m). Spermatogenesis can be intact in mice aged 24 months (Fig. 2 n).

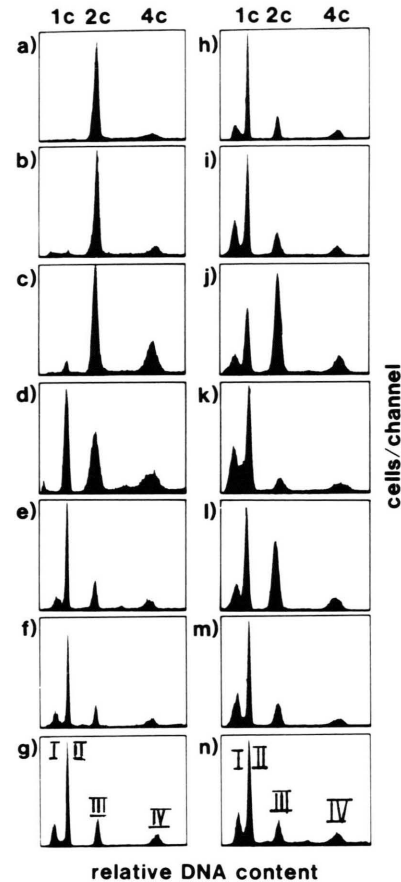


Fig. 2. Representative DNA histograms of mice of different age: a) 0 weeks; b) $1\frac{1}{2}$ weeks; c) 3 weeks; d) $4\frac{1}{2}$ weeks; e) 6 weeks; f) 12 weeks; g) 12 months; h) 18 months; i) 21 months; j, k) 21 months; l, m) 22 months; n) 24 months. About 30 000 cells were measured per histogram. In figures g) and n), the location of the peaks I, II, III and IV representing elongated spermatids, round spermatids, diploid (2c) cells and 4c cells is indicated.

Haploid germ cells

The percentages of round and elongated spermatids as well as the sum of both increase between the 3rd and 6th week of life and stays constant more or less thereafter (Fig. 3 a–c). The increase in number of haploid germ cells parallels the increase in testis weight (*cf.* Fig. 1). The variability of the percentage of haploid germ cells is very low. In 124 mice aged 8 weeks, the arithmetic mean \pm standard deviation ($\bar{x} \pm s$) is 66.8 ± 5.6 %. The region of 61 to 72 % 1c cells is called “normal”

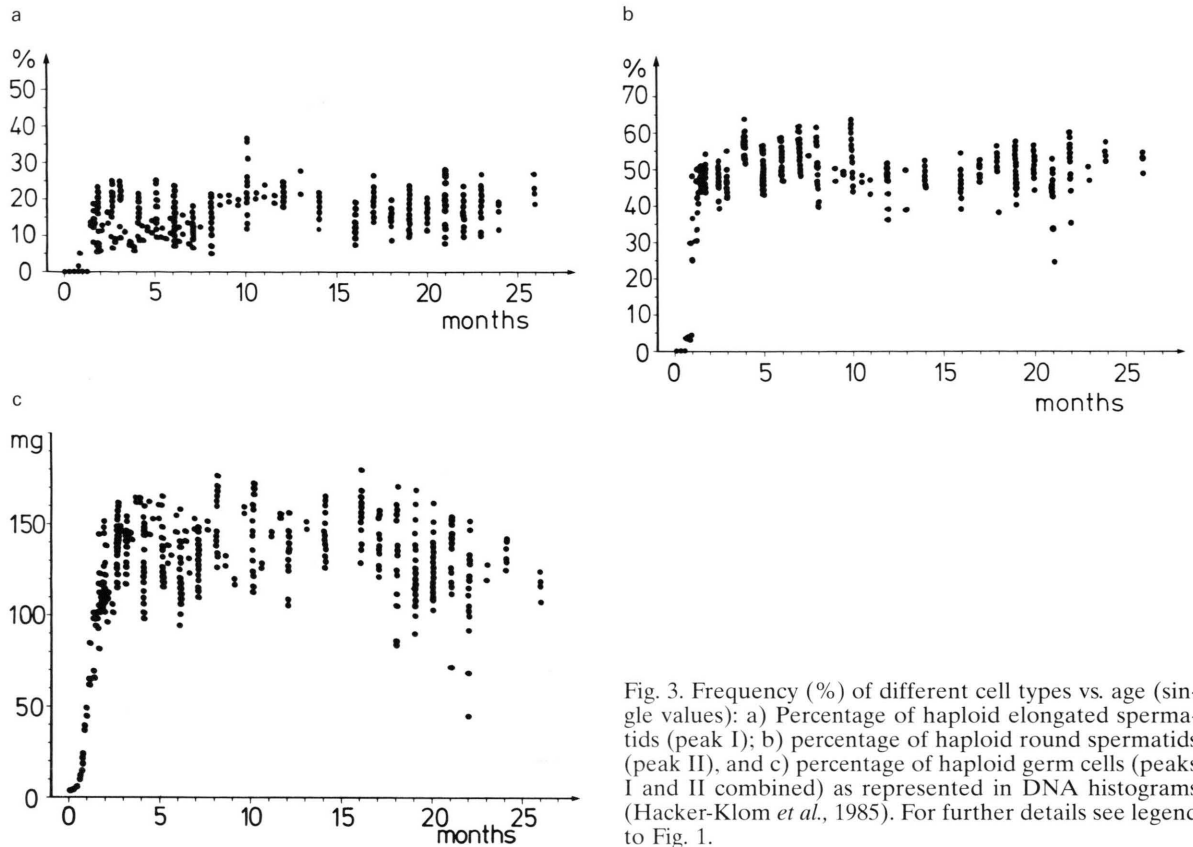


Fig. 3. Frequency (%) of different cell types vs. age (single values): a) Percentage of haploid elongated spermatids (peak I); b) percentage of haploid round spermatids (peak II), and c) percentage of haploid germ cells (peaks I and II combined) as represented in DNA histograms (Hacker-Klom *et al.*, 1985). For further details see legend to Fig. 1.

therefore. The standard deviation is below 10 % of the arithmetic mean in most age groups. The variability of the numbers of elongated spermatids is much larger than that of round spermatids and the combined "haploid cells". Starting with the age of 16 months, there is a tendency to reduced percentage of haploid germ cells (Fig. 3 c) which is more obvious in the data of round spermatids (Fig. 3 b) than in those of the elongated spermatids (Fig. 3 a).

2c cells

The percentage of diploid (2c) cells is 75–80 % at birth, and increases up to 90–95 % of all cells within the first two weeks of life before decreasing (Fig. 4 a). The reason for the relative reduction of 2c cells is the absolute increase of germ cells with a 1c and 4c DNA content in the testes. In the adult mouse, the percentage of 2c cells is 10–20 %. In

some mice above 17 months of age, there are sharp increases of the 2c percentage. In these animals, testis weight and the percentage of 1c cells are decreased at the same time (Figs 1 and 3 c). These mice have a tubular atrophy.

A high 2c cell percentage is always correlated with a low percentage of germ cells in states 1c and 4c.

S-phase cells

The percentage of cells in the phase of DNA synthesis is 10–15 % at birth (Fig. 4 b). It increases between the 2nd and 6th week of life to about 20 % reflecting the increased proliferative activity of spermatogonia. In mice aged 6 weeks and older, the percentage of S-phase cells is 2.5–10 %. Obviously, the variability (s/\bar{x}) is quite high. There is no age dependent reduction of the frequency of DNA synthesizing cells.

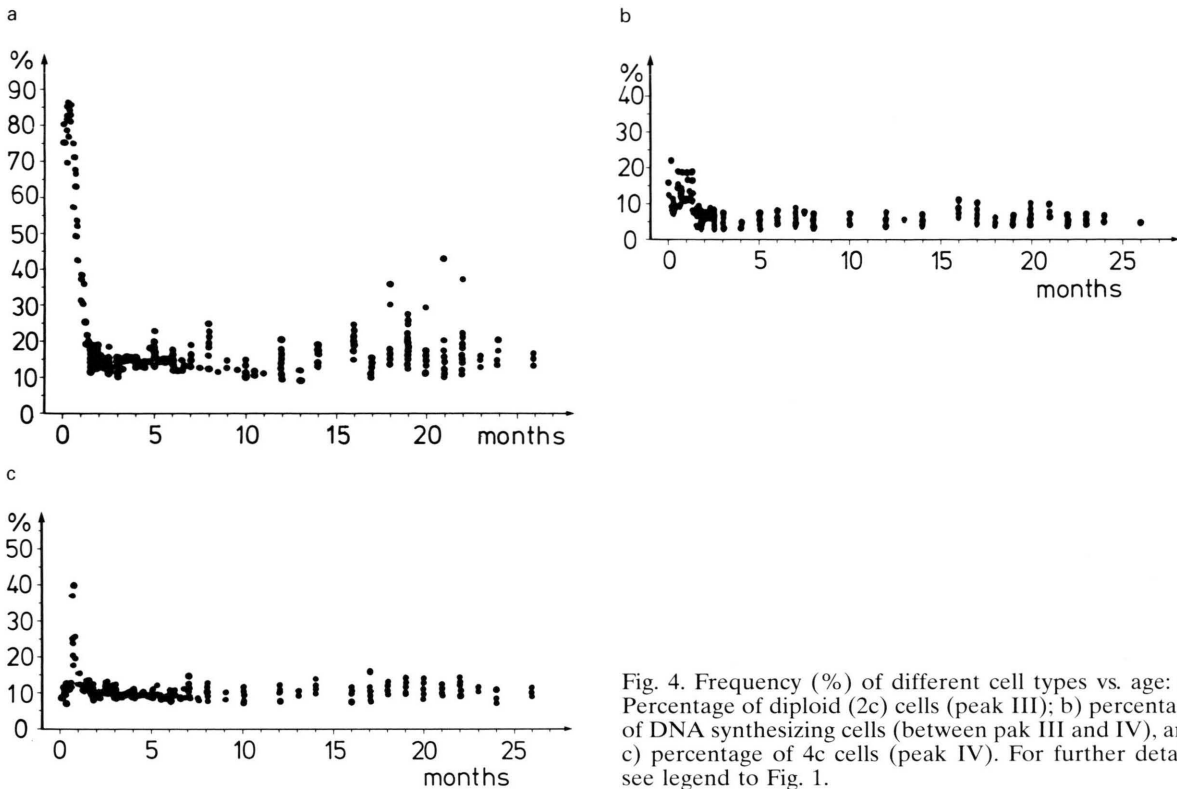


Fig. 4. Frequency (%) of different cell types vs. age: a) Percentage of diploid (2c) cells (peak III); b) percentage of DNA synthesizing cells (between peak III and IV), and c) percentage of 4c cells (peak IV). For further details see legend to Fig. 1.

4c cells

The proportion of 4c cells is about 10 % at birth. In the 3rd week of life, it raises (Fig. 4 c), because of the progressive increase of the numbers of ($G_2 + M$) spermatogonia and primary spermatocytes preceding the increase of haploid germ cells (Fig. 3 c, 4 c). In mice aged 4 weeks and more, the fraction of 4c cells is between 7 and 14 %. No relationship between murine age and the percentage of 4c cells could be detected.

Diploid elongated spermatids

The percentage of diploid elongated spermatids does not increase with age in adult mice (Fig. 5 a).

Coefficient of variation

The ratios between the CV's of peaks I to III resp. of peaks II to III (postmeiotic to premeiotic cells) are calculated in order to have an internal standard (peak III). There is a tendency to an increase of the ratio of CV I/III with increasing

murine age between 2 and 24 months of age (Fig. 5 b). The correlation coefficient is + 0.81. No age dependent increase is detected in the ratio of the CV's of the peaks II and III (Fig. 5 c). The ratios of the CV's of peaks II/III are lowest in mice aged 1 month, 5 and 17 months.

Discussion

The dependence of murine spermatogenesis on the daytime was analysed earlier (Hacker-Klom, 1994). Therefore, in the present paper, the testis samples were taken between 8 a.m. and 12 a.m. resp. 3 p.m. and 5 p.m. when there are no significant changes in the percentages of the different testicular cell types (Hacker-Klom, 1994).

Here, the influence of age on murine spermatogenesis is evaluated in mice aged 0 to 26 months. Round spermatids already appear in mice at the age of $1\frac{1}{2}$ weeks whereas in adult mice, the progression from stem cell spermatogonia to round spermatids takes three weeks (*cf.* Oakberg, 1956).

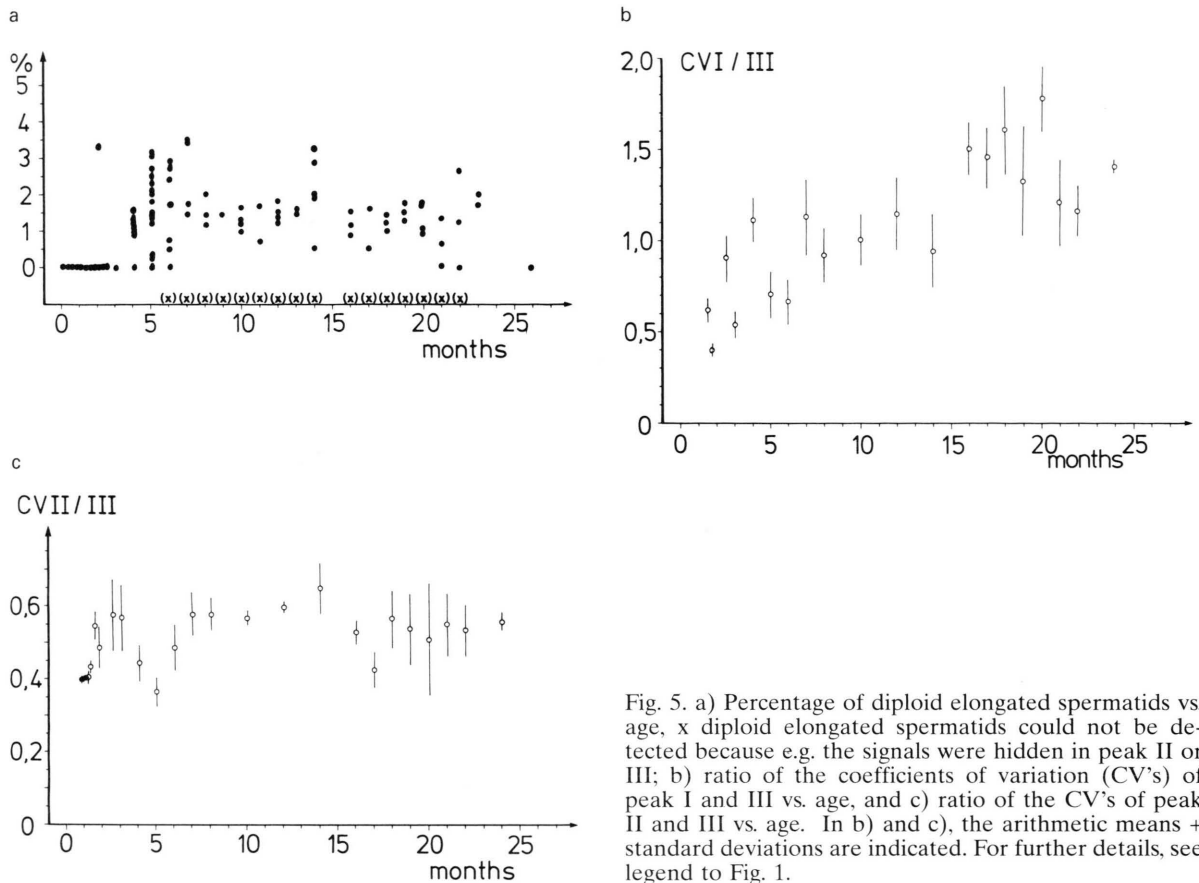


Fig. 5. a) Percentage of diploid elongated spermatids vs. age, x diploid elongated spermatids could not be detected because e.g. the signals were hidden in peak II or III; b) ratio of the coefficients of variation (CV's) of peak I and III vs. age, and c) ratio of the CV's of peak II and III vs. age. In b) and c), the arithmetic means + standard deviations are indicated. For further details, see legend to Fig. 1.

Obviously, spermatogenesis proceeds faster in young than in adult mice. Mice between 11 weeks and 16 months of age show no age dependent changes in testis weight and the number of germ cells and, thus, may be used for experiments on irradiation or drug effects. In mice above 16 months of age, there is a tendency to reduced numbers of spermatogonial cells. Especially the most frequent testicular germ cell type, the round spermatids, and, at the same time, the combined haploid germ cells were reduced in number in mice aged 17 months and more. These changes were paralleled by a decrease of testis weight and an increase of the number of 2c cells reflecting a tubular atrophy. In the frequencies of the other germ cell types (haploid elongated spermatids, primary spermatocytes, spermatogonia), no changes were detected by the experimental and methodological design applied here.

In the present paper, the variability of the numbers of elongated spermatids (peak I) is much larger than that of round spermatids (peak II) and of the combined "haploid cells". Probably, this phenomenon is a consequence of the different status of chromatin condensation in elongated spermatids with differing access for the DNA specific fluorochrome molecules, and the permanent drain of spermatozoa to the epididymis. This higher variability in the frequency of elongated spermatids and spermatozoa might explain why the changes in the percentages of round spermatids with age are more obvious than those of elongated spermatids and spermatozoa.

Suzuki and Withers (1978) found an exponential decrease of stem cell spermatogonia by use of the microcolony assay in unirradiated and irradiated C₃Hf/Bu mice with increasing age between 7 and 122 weeks. This finding indicates that "the lifetime

of the mouse spermatogonial stem cells *in situ* is not uniformly specified but limited in a random manner". In this respect, the conclusion of Suzuki and Withers is in accordance with our results but not with conventional views about stem cells and ageing. Our results, too, may be explained by the random inactivation of stem cell spermatogonia in the testes with age leading to reduced numbers of round spermatids and tubular atrophy in some older mice.

A correlation between age and the width of peak I representing elongated spermatids and spermatozoa as expressed by the ratio of the coefficient of variation (CV) of peak I and III was found ($r = +0.81$). No age-dependent increase was detected in the ratio of peak II (round spermatids) and III. Obviously, a disturbance of chromatin condensation with increasing age is the reason for the increasing variability of peak I representing elongated spermatids, not a real increase of chromosome aberrations.

Thus, our data which were obtained in male NMRI mice are in contradiction to those of Fabricant and Parkening (1982): They found that 12 and 18 months old C557BL/6 mice had a significantly higher frequency of chromosomal aberrations in male germ cells compared to 2 months old animals what is consistent with a theory of decreased repair capacity in cells from aged animals (Alexander, 1967). The frequency of morphologically abnormal spermatozoa is increasing with age in 2 to 25 months old C557BL/6 mice at about a factor of two (Fabricant and Parkening, 1982). Age-dependent changes in morphological characteristics of murine spermatozoa were accompanied by decreased motility and fertilizing ability (Martin-DeLeon and Boice, 1982). But since the assumption of a general correlation between morphology and chromosomal aberrations of murine sperma-

tozoa is a matter of debate (Wyrobek *et al.*, 1979), the data of these authors cannot be correlated with our own data.

Interestingly, no age dependent increase of the frequency of diploid spermatozoa was detected. Considering the involvement of diploid spermatozoa in triploid zygotes – trisomy is the most frequent chromosomal anomaly in mouse and rabbit (Martin-DeLeon and Boice, 1982) – this finding indicates that ageing spermatozoa may play a minor role in the induction of trisomy.

Whether the extreme small ratio of the CV of peaks II and III in mice aged 1 month, 5 and 17 months in our data has seasonal reasons must be investigated further: These samples were obtained in autumn (September/October), and possibly reflect a low testicular activity. The other samples were taken in spring, summer or winter.

In conclusion, it is important to control the age of experimental mice used to study spermatogenesis. Mice aged 11 weeks to 16 months are suitable for studies on other than age effects on spermatogenesis. Since in these age groups, there also were no significant changes between the germ cell percentages of both testes of the mice, e.g. the left testis might serve as an internal control for the right testis if treated non systemically.

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- Alexander P. (1967), The role of DNA lesions in the processes leading to aging in mice. *Symp. Soc. Biol.* **21**, 29–50.
- Fabricant J. D. and Parkening T. A. (1982), Sperm morphology and cytogenetic studies in ageing C 57 Bl/6 mice. *J. Reprod. Fert.* **66**, 485–489.
- Hacker-Klom U. (1994), Diurnal changes in murine spermatogenesis. *Z. Naturforsch.* **49c**, 522–525.
- Hacker-Klom U., Heiden T., Mauro F. and Göhde W. (1989), Radiation-induced diploid spermatids in mice. *Int. J. Radiat. Biol.* **55**, 797–806.
- Kovaleva N. V. (1992), Age and chromosome nondisjunction. *Genetika* **28**, 154–161.
- MacLeod J. and Gold R. Z. (1953), The male factor in fertility and infertility. VII. Semen quality in relation to age and sexual activity. *Fert. Steril.* **4**, 10–33.
- Martin-DeLeon P. A. and Boice M. L. (1982), Sperm aging in the male and cytogenetic anomalies. An animal model. *Hum. Genet.* **62**, 70–77.
- Murdock J. L., Walker B. A. and McKusick V. A. (1972), Parental age effects on the occurrence of new mutations of the Marfan syndrome. *Ann. Hum. Genet.* **35**, 331–336.
- Meier E. M. (1983), Zellbiologischer Informationsgehalt der DNS-Verteilungen von Säugetierzellen und menschlichen Spontantumoren. Inaugural-Dissertation, Universität Münster.
- Meistrich M. L. and Samuels R. C. (1985), Reduction in sperm level after testicular irradiation of the mouse. A comparison with man. *Radiation Res.* **102**, 138–147.
- Oakberg E. F. (1956), Duration of spermatogenesis in the mouse and timing of the stages of the seminiferous epithelium. *Am. J. Anat.* **99**, 507–516.
- Otto F. J. and Oldiges H. (1980), Flow cytogenetic studies in chromosomes and whole cells for the detection of clastogenic effects. *Cytometry* **1**, 13–17.
- Otto F. J. and Oldiges H. (1986), Entwicklung einer durchflußzytometrischen Methode für Mutagenitätsprüfungen an Keimzellen der Maus. *Wissenschaft und Umwelt* **1**, 15–30.
- Regaud C. and Ferroux R. (1927), Discordance des effets des rayons X d'une part dans le peau, d'autre part dans le testicule, par le fractionnement de la dose, diminution de l'efficacité dans le peau, maintien de l'efficacité dans le testicule. *CR Soc. Biol.* **97**, 431–447.
- Smith C. A. B. (1972), Note on the estimation of parental age effects. *Ann. Hum. Genet.* **35**, 337–342.
- Sora S. and Magni G. E. (1982), Meiotic diploid progeny and meiotic non-disjunction in *Saccharomyces cerevisiae*. *Genetics* **101**, 17–33.
- Suzuki N. and Withers H. R. (1978), Exponential decrease during aging and random life time of mouse spermatogonial stem cells. *Science* **202**, 1214–1215.
- Tatchen I., Hacker-Klom U. and Göhde W. (1989), Flow cytometry and sorting of diploid elongated spermatids and other testicular cell types in the mouse. Poster, 1st Meeting of the European Society for Analytical Cellular Pathology, Schloß Elmau, Bayern.
- Tunte W., Becker P. E. and Von Knorre G. (1967), Zur Genetik der myositis ossificans progressiva. *Human-genetik* **4**, 320–351.
- Wyrobek A. J. (1979), Changes in mammalian sperm morphology after X-ray and chemical exposure. *Genetics* **92**, suppl., s105-s119.