Quantitation of spermatogenesis by DNA flow cytometry: Comparative study among six species of mammals

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Abstract. Suspensions of testicular germ cells from six species of mammals were prepared and stained for the DNA content with a fluorochrome (ethidium bromide) adopting a common technique and subjected to DNA flow cytometry. While uniform staining of the germ cells of the mouse, hamster, rat and monkey could be obtained by treating with 0·5% pepsin for 60 min followed by staining with ethidium bromide for 30 min, that of the guinea pig and rabbit required for optimal staining pepsinization for 90 min and treatment with ethidium bromide for 60 min. The procedure adopted here provided a uniform recovery of over 80% of germ cells with each one of the species tested and the cell population distributed itself according to the DNA content (expressed as C values) into 5 major classes– spermatogonia (2C), cells in S-phase, primary spermatocytes (4C), round spermatids (1C), and elongating/elongated spermatids (HC). Comparison of the DNA distribution pattern of the germ cell populations between species revealed little variation in the relative quantities of cells with 2C (8–11%), S-phase (6–9%), and 4C (6–9%) amount of DNA. Though the spermatid cell populations exhibited variations (1C:31–46%, HCl:7–20% and and HC2:11–25%) they represented the bulk of germ cells (70–80%). The overall conversion of 2C to 1C (1C:2C ratio) and meiotic transformation of 4C cells to 1C (1C:4C ratio) kinetics were relatively constant between the species studied. The present study clearly demonstrates that DNA flow cytometry can be adopted with ease and assurance to quantify germ cell transformation and as such spermatogenesis by analysing a large number of samples with consistency both within and across the species barrier. Any variation from the norms in germ cell proportions observed following treatment, for *e.g.* hormonal stimulation or deprivation can then be ascribed due to a specific effect of the hormone/drug on single/multiple steps in germ cell transformation.

Keywords. Testicular germ cells; mammals; DNA flow cytometry; germ cell transformation kinetics.

1. Introduction

In the field of male reproductive biology there is an acute need for a suitable method which can provide rapid, sensitive, and quantitative measurement of various testicular germ cell types. Such information could help assess the status of spermatogenic process under various experimental/pathological conditions including that produced by a contraceptive under test. Flow cytometry combines many of the advantages of microscopy and biochemical analysis in a single high precision technique for rapid phenotypic and genotypic analysis of individual cells using a limited quantity of the tissue as available at biopsies. Since the DNA content of the germ cells changes distinctly during the various steps of proliferation and transformation in spermatogenesis, DNA flow cytometry offers a method to

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monitor with relative ease such changes in dispersed testicular cells. Flow cytometric analysis of testes of the mouse (Toppari *et al* 1988), rat (Clausen *et al* 1978a, b, 1979, 1982; Toppari *et al* 1986; Van Kroonenburgh *et al* 1986; Vaishnav 1988), monkey (Aravindan *et al* 1990) and the human (Thorud *et al* 1980) has been carried out but the methods of cell preparation, fluorochromes/stains used and the flow cytometer employed as well have been different. The present study was undertaken with a view to determine the extent to which the technique developed in our laboratory for one species (i.e., the monkey) using a Beckton- Dickinson flow cytometer (FACScan) for analysis and ethidium bromide (EB) to fluorescence tag the DNA can be adopted to the other commonly used laboratory species of mammals, and whether the germ cell preparation of different species show any distinct variation in the per cent germ cells recovered and their transformation ratios.

2.   Materials and methods

2.1   *Animals*

Five sexually mature adult males from each of the following species were included in the study: mouse, rat, hamster, guinea pig, rabbit and monkey. Except bonnet monkeys (*Macaca radiata*) which were from the colony maintained at the Primate Research Laboratory, the rest of the species were obtained from the Central Animal Facility of the Institute. All the animals were maintained under a regulated light: dark (12:12h) schedule with food and water provided *ad lib*.

2.2   *Chemicals and solutions*

Dulbecco's phosphate buffered saline (PBS, pH 7·4) was obtained from Hi-media, Bombay, India; pepsin from Serva Heidelberg, Germany; EB, ribonuclease (RNase) and Nonidet p-40 (NP-40) from Sigma Chemical Co., St. Louis, Mo, USA; Ketamine hydrochloride was from Parke-Davis, Detroit, MI, USA, and Sodium pentabarbitone from May and Baker, England.

2.3 *Germ cell preparation*

The testicular tissue was obtained either by biopsy (monkeys) or by unilateral castration (rabbits), or by euthenising the animals. Care was taken to select the same region of the testis (contralateral to the epididymis) while removing the tissue in all the instances. The testicular tissue, ranging from 50–100 mg was immediately transferred to a petridish containing a small volume of PBS, finely minced, passed gently through the hub of a syringe and then vortexed briefly to maximise the release of germ cells from the seminiferous tubule minces. The cell suspension was then filtered using a nylon filter (Nybolt P-25, Swiss Silk, Zurich, Switzerland) and a drop of the filtrate was observed under the microscope to check the number and integrity of the released germ cells before fixing in 80% chilled ethanol and storing at 4° C until analysis.

2.4 *DNA staining and flow cytometry*

The staining procedure followed was essentially similar to that routinely used for the monkey germ cells in our laboratory except for different durations of treatment. Briefly, the fixed cells were washed with the buffer and equal concentration of cells was taken and incubated with 0·5% pepsin in isotonic PBS (pH 2·0) for 60, 90 or 120 min at 37° C. After centrifugation at 900 g for 10 min, to the cell pellet was added 200 μ1 of staining solution (25 μg EB, 0·004% RNase and 0·3% NP-40 in PBS, final pH 7·4) and left in the dark at room temperature for 30, 45, or 60 min. Treating with pepsin beyond 120 min resulted in the loss of population identity in the flow cytogram. The concentration of RNase used here is far in excess of that used by Toppari *et al* (1988, 5 μg/ml) and has been found to be adequate to remove any contaminating RNA that may interfere with the coefficient of variation of the DNA peaks analysed. The flow cytometer used was a FACScan (Beckton-Dickinson) equipped with an air-cooled 15 milliwatt Argon-Ion laser at 488 nm and the emitted light from the cell samples (EB stained) was detected at 545 nm on FL2. Uniform number of cells (10,000/sample at a flow rate of 600–700 events/s) were acquired and analysed for the per cent germ cell populations and their transformation kinetics, expressed as ratios. The different populations of germ cells thus obtained based on the relative fluorescence intensities of their DNA content were expressed as C values using human peripheral blood leucocytes as the diploid standard (Otto *et al* 1984). The values are expressed as mean ± SEM.

3. Results

Five different populations of germ cells characteristic of sexually mature adult testis could be identified in all the species studied (figure 1). Based on the relative fluorescence intensities of the DNA content the cell populations were classified as spermatogonia (2C), pre-leptotene spermatocytes (S-phase), primary spermatocytes (4C), round spermatids (1C), and elongating (HCl) and elongated (HC2) spermatids. Once the location of 2C population of cells in the flow cytogram is arrived at by analysing flow cytometrically a human peripheral lymphocyte preparation, the 1C and 4C populations were ascertained on the corresponding fluorescence intensities of their DNA contents.

   Of the various species tested, the mouse, rat, hamster and monkey germ cells required a 60 min pepsinization followed by a 30 min exposure to EB to provide an optimal flow cytogram. That of guinea pig and rabbit, however, required a longer period of treatment (pepsinization/EB exposure–90/60 min) to provide a comparable flow cytogram (figure 1).

   Of the total of 10,000 cells acquired per sample approximately 83% (83·21 ± 4·22– SD) constituted the various populations of germ cells that could be clearly discernible indicating that the recovery of germ cells by the procedure adopted here is fairly uniform, reproducible and good (table 1). In all cases the germ cells could primarily be categorised under two types — those having 1C amount of DNA represented in the flow cytogram as 1C, HCl and HC2 (70–80% of the total population) and those having 2C to 4C (20–30%) amount of DNA, The latter is seen as two distinct peaks — 2C and 4C, the cells in between these two peaks being
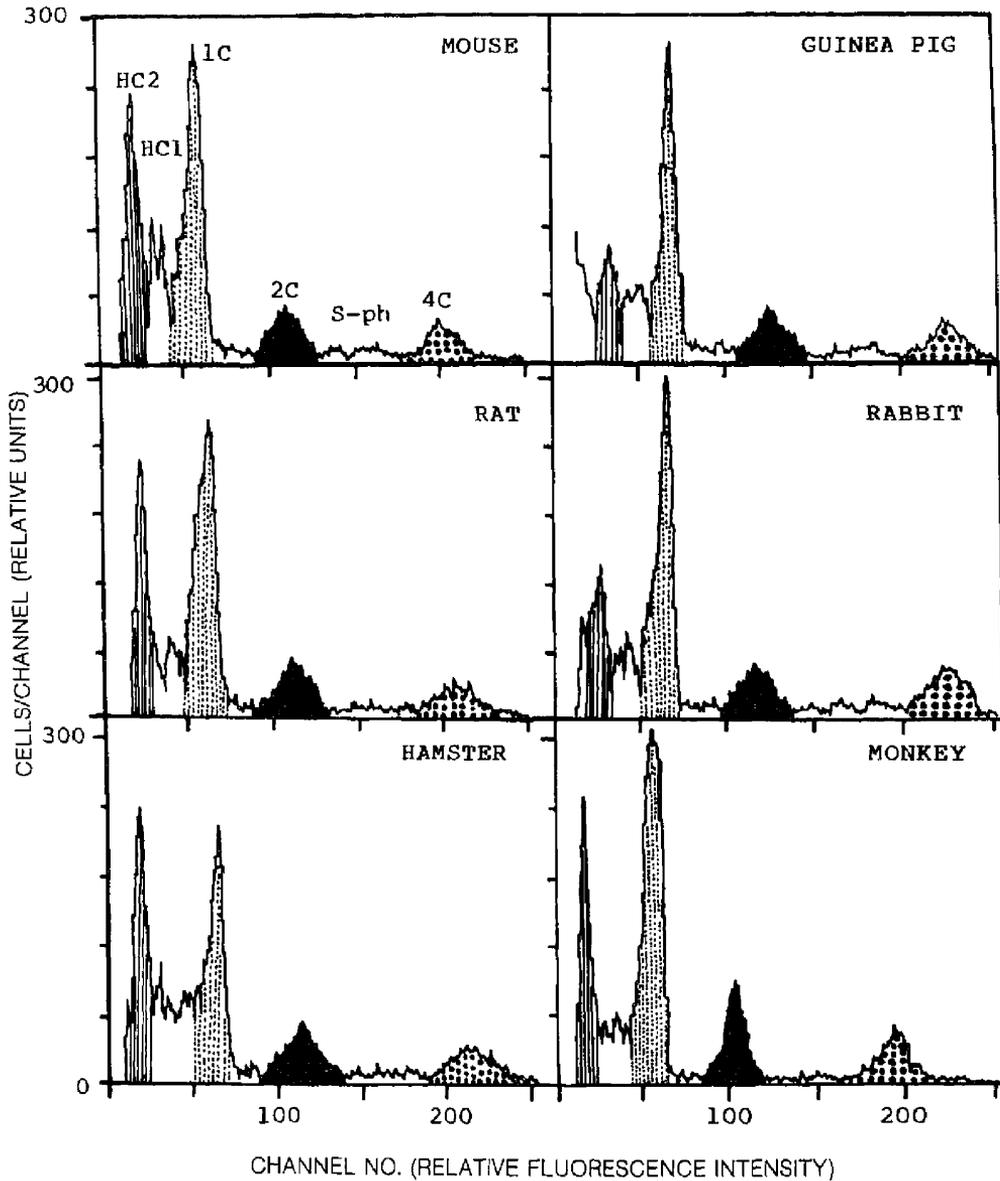
Figure 1. Representative flow cytograms showing the distribution of testicular germ cell populations based on their DNA content in the six species of mammals studied by DNA flow cytometry.

characterized as those in S-phase (figure 1) The categorization of HCl and HC2 is based on the differences in fluorescence intensities of the spermatid populations appearing before the 1C peak indicating that due to continued compaction spermatid DNA undergo during spermiogenesis, they bind lesser than normal amount of EB (Clausen *et al* 1982).

The 2C population of cells which might include besides spermatogonia a small

Table 1. Per cent testicular germ cell populations in six species of mammals analysed by DNA flow cytometry.

Species*	Total % GC recovered/ 10,000 cells	Germ cell populations (%)[§]							
		Haploids				Non-haploids			
		HC2	HC1	1C	Total	2C	S-phase	4C	Total
Mouse	89·3	24·3	16·3	39·0	79·8	8·5	6·1	5·8	20·2
(*Mus musculus*)	± 0·5	± 1·6	± 1·8	± 2·1	± 1·9	±0·5	±0·5	±0·3	± 0·8
Rat	83·1	20·2	18·4	38·4	77·0	8·2	7·6	7·2	23·0
(*Rattus norvagicus*)	± 1·7	± 1·6	± 1·4	± 1·4	± 2·1	±0·6	±0·7	±0·5	± 0·3
Hamster	86·3	26·8	17·4	31·1	75·3	9·0	7·9	7·8	24·7
(*Mesocricetus auratus*)	± 2·5	± 2·0	± 3·4	± 2·6	± 1·6	±1·1	±0·9	±0·9	± 1·2
Guinea pig	77·3	11·2	18·3	43·7	73·2	9·5	9·3	7·0	25·8
(*Cavia porcellus*)	± 3·0	± 3·1	± 2·9	± 2·7	± 2·3	±0·1	±1·2	±0·3	± 1·6
Rabbit	80·5	13·3	19·3	39·4	72·0	10·8	8·9	8·3	28·0
(*Oryctolagus cuniculus*)	± 1·4	± 2·2	± 1·6	± 1·3	± 1·6	±0·4	±0·9	± 0·3	± 1·1
Monkey	82·8	20·4	7·8	46·4	74·6	9·8	7·4	8·2	25·4
(*Macaca radiata*)	± 1·2	± 1·4	± 0·4	± 1·8	± 2·1	±0·4	±1·0	±0·6	± 1·5

*n = 5/species.
[§]Germ cell populations were calculated considering the total % recovery values as 100%. Values are Mean ±SEM.

population of somatic cells (Leydig/Sertoli cells) that may get liberated during cell preparation procedure adopted accounted in all species to 10% or less (table 1). The spermatocyte population (4C) as well as the cells in S-phase also showed a remarkable uniformity across the species barrier (table 1). The transformation kinetics of germ cell populations is presented as ratios in table 2. The 1C:2C ratios which provide the overall turnover of spermatogonial cells to round spermatids are relatively constant between species. The 4C: 2C, 4C: S-phase, and 1 C: 4C ratios also show reasonable consistency. The variations seen in the ratios of HC2:1C (elongated: round spermatids) and HC2:HC1 (elongated: elongating spermatids) probably reflect the differences in the rate of transformation of the round spermatids to elongating/elongated spermatids between species.

Table 2. Kinetics of germ cell transformations (ratios) in the six species of mammals studied by DNA flow cytometry.

Species*	Germ cell ratios					
	1C:2C	4C:2C	4C:S-ph	1C:4C	HC2:1C	HC2:HCl
Mouse	4·64±0·26	0·68±0·02	0·93±0·09	6·84±0·50	0·63±0·05	1·62±0·24
Rat	4·68±0·19	0·87±0·01	0·83±0·09	5·33±0·30	0·53±0·05	1·09±0·10
Hamster	3·50±0·24	0·86±0·04	0·98±0·13	4·01±0·45	0·86±0·40	1·54±0·21
Guinea pig	4·60±0·44	0·73±0·03	0·75±0·09	6·24±0·43	0·26±0·08	0·62±0·19
Rabbit	3·70±0·12	0·77±0·03	0·93±0·08	4·96±0·13	0·34±0·06	0·69±0·15
Monkey	4·70±0·70	0·83±0·09	1·06±0·17	5·66±0·87	0·43±0·01	2·61±0·30

*n= 5/species
Values are mean ± SEM.

4. Discussion

The primary objective of this investigation was to determine if the procedure worked out for analysing testicular germ cells of one species by flow cytometry is easily adaptable for other laboratory animal species. This has been adequately demonstrated by the results of the current study. It is interesting that the results of the present study are essentially similar to that reported earlier by us for the rat (Vaishnav *et al* 1988) and the monkey (Aravindan *et al* 1990) using a different fluorochrome (DAPI) and a different flow cytometer (Phywe ICP-22, Phywe A G, Goettingen, Germany). Though the rate of transformation of one germ cell type to another in different species appears similar, they also exhibit subtle differences (for *e.g.* in spermiogenesis - HC2:1C and HC2: HC1 ratios) and this may reflect differences between the species in the time taken for completion of one spermatogenic cycle. Several recent studies by our group (Vaishnav *et al* 1988; Moudgal *et al* 1988; Aravindan and Moudgal 1991; Aravindan *et al* 1991; Suresh and Moudgal 1992; Jeyakumar *et al* 1992) as well as others (Isoyama 1986; Weinbauer *et al* 1991) have shown using DNA flow cytometric analytical procedures that testicular germ cell transformations are amenable to alteration by specific hormonal deprivation or stimulation suggesting thereby that this would be an efficient and accurate method to investigate problems on regulation of spermatogenesis. As the results obtained across the species barrier as demonstrated here are uniform and reproducible, any changes observed in germ cell transformation as a consequence of hormonal stimulation/deprivation or drug treatment will be significant and contribute to the understanding of the action of the hormone/drug. We have, in addition, noted that the DNA distributions from biopsy samples taken from six different loci on the same testis of a monkey reveal no significant differences in the per cent germ cell populations of 2C (10–12%), S-phase (5–7%) and 4C (6–8%). The spermatid population, however, exhibited some variation (1C:36–46%; HCl: 18–28%; HC2:9–14%) between the regions. Such regional differences in the spermatid population have also been reported following analysis of biopsy samples from the human testis (Clausen *et al* 1978b). Besides, the kinetics of germ cell transformation showed little variation between the anterior, posterior, right, and left regions of the testis where as the dorsal and ventral regions showed minor differences in the 1C:2C and 1C:4C ratios. Based on the observations of Thorud *et al* (1980) and our own observations in the rabbit (Jeyakumar *et al* 1992) and monkey (Suresh and Moudgal 1992), it is clear that flow cytometric analysis of one or two biopsy samples could be adequate to provide a true picture of the status of spermatogenic activity. Besides species as well as sexual maturity of the test animal determining the specific hormonal effect, it is becoming increasingly clear that hormones like FSH and testosterone regulate spermatogenesis by influencing transformation of specific but different germ cell types (Suresh and Moudgal 1992; Jeyakumar *et al* 1992). In infertile men, therefore, a pretherapy flow cytometric analysis of testicular germ cell population may help determine the lesion and the therapy that has to be resorted to restore normal spermatogenesis.

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