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Selective immunoneutralization of luteinizing hormone results in the apoptotic cell death of pachytene spermatocytes and spermatids in the rat testis

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The selective withdrawal of pituitary gonadotropins through specific antibodies is known to cause disruption of spermatogenesis. The cellular mechanism responsible for the degenerative changes under isolated effect of luteinizing hormone (LH) deprivation is not clear. Using antibodies specific to LH we have investigated the effect of immunoneutralization of LH on apoptotic cell death in the testicular cells of the immature and the adult rats. Specific neutralization of LH resulted in apoptotic cell death of germ cells, both in the immature and the adult rats. The germ cells from control animals showed predominantly high molecular weight DNA, while the antiserum treated group showed DNA cleavage into low molecular weight DNA ladder characteristic of apoptosis. This pattern could be observed within 24 h of a/s administration and the effect could be reversed by testosterone. The germ cells were purified by centrifugal elutriation and the vulnerability of germ cell types to undergo apoptosis under LH deprivation was investigated. The round spermatids and the pachytene spermatocytes were found to be the most sensitive germ cells to lack of LH and underwent apoptosis. Interestingly, spermatogonial cells were found to be the least sensitive germ cells to the lack of LH in terms of apoptotic cell death. Results show that LH, in addition to being involved in the germ cell differentiation, is also involved in cell survival and prevent degeneration of germ cells during spermatogenesis. Apoptotic DNA fragmentation may serve as a useful marker for the study of hormonal regulation of spermatogenesis and the specific neutralization of gonadotropic hormones can be a reliable model for the study of the molecular mechanism of apoptosis.

Keywords: immunoneutralization; luteinizing hormone; apoptosis; testis; pachytene spermatocytes; spermatids

Introduction

Pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) are the principal regulators of the germ cell differentiation in the testis. Both these hormones regulate spermatogenesis by regulating the function of the Sertoli cells, FSH directly and LH indirectly through testosterone produced by the Leydig cells produced in response to the hormone. There have been many recent studies focusing on the spontaneous wasting of germ cells during spermatogenesis, evaluated mainly by the morphological analysis (Allan *et al.*, 1992 and Johnson *et al.*, 1983; Miething, 1992) and the mechanism for this has been conclusively identified as the programmed cell death or apoptosis. This process appears to be an efficient mechanism for eliminating unwanted cells in the midst of living tissues, especially under conditions of hormone withdrawal. The withdrawal of hormone was shown to result in the apoptosis of many reproductive tissues such as prostate (Buttyan *et al.*, 1989), Ovary (Tilly *et al.*, 1991) and testis (Tapanainen *et al.*, 1993 and Troiano *et al.*, 1994). It has been well established through the work carried out by our group that the blockade in the action of hormones by specific antibodies leads to the disruption of germ cell differentiation in the testis (Moudgal and Dighe, 1985; Murty *et al.*, 1979; Sheela Rani *et al.*, 1978; Shivashankar *et al.*, 1977; Vaishnav and Moudgal, 1991). We speculated that this disruption could be due to the degeneration of the germ cells taking the course of programmed cell death. This suggested that the gonadotropins, in addition to being responsible for differentiation of the germ cells, are also involved in the survival of the germ cells in the testis. Further, it also suggests that germ cell death could be amplified by removing specific trophic hormones. The phenomenon of apoptosis can be a sensitive marker to study the role of gonadotropins in spermatogenesis and also to identify the cells that are most sensitive to lack of individual gonadotropins. It is known that the nuclear changes in an apoptotic cell are caused by activation of an endogenous calcium-magnesium sensitive nuclease which cleaves the chromatin between nucleosomes, reducing the DNA to a series of fragments, integer size multiples of 180–200 base pairs, and thus producing a characteristic 'ladder' on agarose gel electrophoresis (Wyllie, 1993). In the present communication an attempt has been made to identify the LH sensitive cells in the testis by neutralizing the hormone by specific antibodies, purifying the different germ cells by centrifugal elutriation and demonstrating the antibody induced apoptotic DNA fragmentation in purified germ cells.

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Results

Characterization of oLH antiserum

The oLH a/s used in the study showed 50% binding with ^{125}I rat LH at 1:20,000 dilution. In adult rats, 300 µl of this antiserum was able to significantly reduce the circulating testosterone level 3 h after the administration of the antiserum (control: 2.36 ± 0.65 ng/ml; LH a/s treated: 0.81 ± 0.41 ng/ml). In case of immature animals, the serum testosterone levels were undetectable (less than 0.1 ng/ml) as compared to 1 ± 0.3 ng/ml in controls ($n = 6$). Further, 75 µl of the antiserum administered for 7 days in 21 day old rats was able to bring about 55% decrease in the relative weight of the paired testes (control: 568 ± 73 mg/pair of testes; LH a/s treated: 253 ± 24 mg/pair of testes).

Induction of apoptotic DNA fragmentation in the rat testis by LH a/s

Immature 21 day old rats were treated with oLH antiserum (75 µl/rat/day) for one or two days with a gap of 24 h in between 2 injections. The animals were sacrificed 24 h after the last antiserum injection. DNA was isolated from the total

testicular tissue and electrophoresed on 2% agarose gel. It is evident from the ethidium bromide stained gel shown in the Figure 1 that while the DNA from control rat testis show high molecular weight DNA, the DNA isolated from antiserum treated animals show DNA laddering characteristic of apoptotic DNA fragmentation. This fragmentation could be seen within 24 h of the antiserum treatment but was more pronounced in 48 h treatment group.

Reversal of apoptotic DNA fragmentation by testosterone

In order to find out if the effect of a/s can be reversed by simultaneous administration of testosterone, the animals were administered testosterone 3 mg/rat/day along with the antiserum as above and sacrificed after 24 h of second injection. DNA was isolated from the total testis and electrophoresed on 2% agarose gels. As shown in Figure 2, apoptotic DNA fragmentation can be seen upon antiserum administration. However with simultaneous testosterone administration DNA fragmentation appeared to be less, although not completely abolished suggesting that testosterone can partially reverse the apoptotic DNA fragmentation in the testicular germ cells induced by LH a/s.

Identification of cell types most sensitive to lack of gonadotropins

Having demonstrated the effect of the antiserum on DNA fragmentation on total testicular germ cells, it was of interest to find out the cell types which are most sensitive to lack of gonadotropins. For this purpose, oLH antiserum was administered to either immature or adult animals as described above for 2 days and sacrificed 24 h after the second injection. The different population of the germ cells were purified by centrifugal elutriation. The purity of each cell preparation was determined by DNA flow cytometry and staining of the cells. DNA from each cell type was isolated and electrophoresed on 2% agarose gels. Figure 3(I) shows the DNA from the individual cell preparations from the immature rats treated with oLH a/s. The lane 2 shows the DNA from the entire germ cell population from control testis, while the lane 3 shows the DNA from similar cells obtained from the testis of LH a/s treated rats. The Lane 4 shows the DNA from spermatogonia enriched fraction while the lane 5 shows the DNA from pachytene enriched populations isolated from the a/s treated group. As is evident from these data, only the DNA from pachytene enriched populations show apoptotic DNA fragmentation. The purity of each enriched fraction is shown in the Figure 3(II).

Figure 4 shows the DNA pattern of the cell populations isolated from the control and LH a/s treated adult rats. It is clear from the data presented that while DNA from round spermatids and pachytene enriched control cells show predominantly high molecular weight DNA, the DNA from LH a/s treated show DNA laddering characteristic of apoptosis (Figure 4(I)). Figure 4 (II) shows the purity of each kind of germ cell population used for DNA isolation.

Discussion

Although it is clearly accepted that the pituitary gonadotropins play a significant role in the process of the germ cell differentiation, their precise biochemical roles have not been delineated. The recent observation that hypophysectomy and

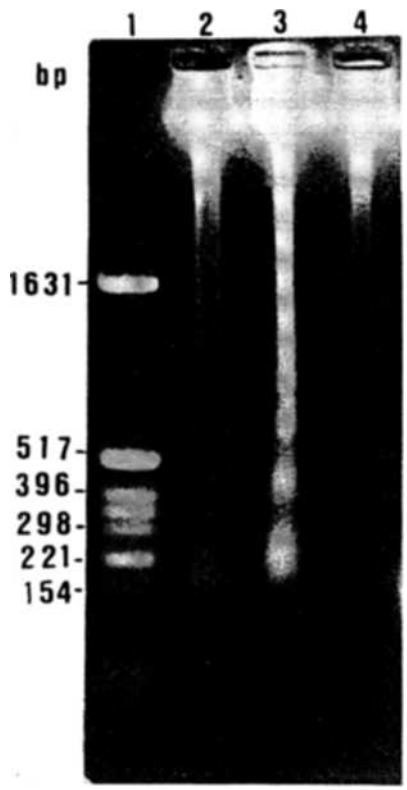

Figure 1 Effect of LH antiserum treatment on testicular DNA fragmentation. Twenty-one day old rats ($n = 6$ per group) were administered oLH antiserum for one or two days. Twenty-four hours after each injection the animals were sacrificed, DNA was isolated from the testis and electrophoresed on 2% agarose gel. Lane 1: pBR 322 Hinf markers; Lane 2: LH a/s 24 h; Lane 3: LH a/s 48 h; Lane 4: control.

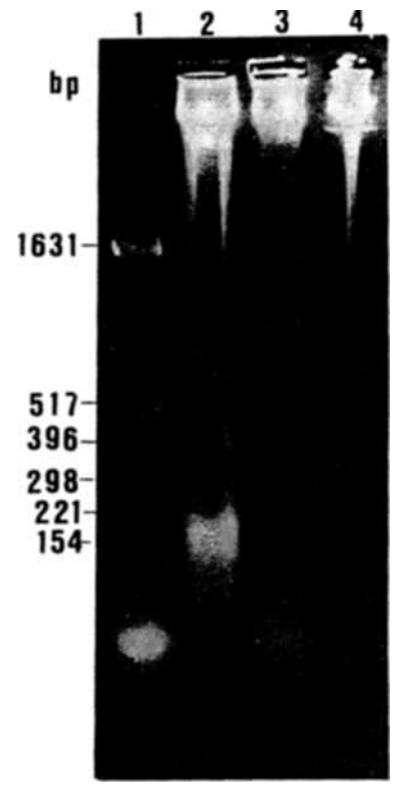

Figure 2 Reversal of LH antiserum induced testicular DNA fragmentation by testosterone. Twenty-one day old rats ($n = 6$ per group) were treated with oLH antiserum as described in Figure 1. Testosterone (3 mg) was also administered to the animals along with the antiserum. The animals were sacrificed, testicular DNA extracted and electrophoresed on 2% agarose gel. Lane 1: pBR 322 Hinf markers; Lane 2: LH a/s 48 h; Lane 3: LH a/s 48 h + testosterone; Lane 4: Control.

administration of GnRH antagonists results in programmed cell death in the germ cells which exhibit characteristic DNA fragmentation has led to the hypothesis that the gonadotropins are also involved in the survival of the germ cells. (Tapanainen *et al.*, 1993; Billig *et al.*, 1995). Further, Troiano *et al.* (1994), have shown that testosterone withdrawal leads to the apoptosis of testicular germ cells. Use of specific antibodies has allowed us to define the roles of the individual gonadotropins in the process of gonadal differentiation (Moudgal and Dighe, 1985). With availability of the new sensitive marker, apoptotic DNA fragmentation, it has been possible for us to clearly demonstrate the role of LH in the survival of the germ cells.

Administration of LH antibodies to immature 21 day old rats resulted in the arrest of spermatogenesis. We also demonstrated that neutralization of LH, which resulted in almost 100% decrease in serum testosterone, causes decrease in spermatogonial proliferation and prevents the entry of cells into meiosis (Shetty *et al.*, 1994, communicated). In the present study, we have demonstrated that the deprivation of LH results in the apoptotic DNA fragmentation in the testicular germ cells of 21 to 23 day old rats. This effect is partially reversible by testosterone. The cells most affected by lack of LH in the immature germ cells were the pachytene spermatocytes. This observation is in agreement with the findings of Billig *et al.* (1985). However they postulated that LH plays less important role than FSH, the other gonadotropic hormone, in the germ cell survival. But our observations clearly demonstrate the involvement of LH as a germ cell survival factor, although it is indirect and mediated by testosterone. Tapanainen *et al.* (1993), using hypophysectomized rats, demonstrated the suppressive effect of testosterone on apoptotic cell death. Our observations confirm that testosterone is definitely involved in mediating the suppression of cell death. The spermatogonia enriched population did not show appreciable DNA fragmentation. Interestingly, in an another study we demonstrated that rate of DNA synthesis by the spermatogonia enriched fraction from the LH a/s treated rats was significantly less compared to that observed by similar cells from the controls (Shetty *et al.*, 1994; communicated). This suggests that deprivation of LH does not result in the apoptotic death of spermatogonia, but their differentiation is definitely affected. It may be postulated that LH deprivation has a differential effect on the premeiotic and postmeiotic germ cells. It is clear from the present studies that the germ cells become vulnerable to undergo apoptosis under LH deprivation only after they enter meiosis.

The results of the experiments with the adult rats clearly indicated the sensitivity of spermatids and pachytene spermatocytes to undergo apoptosis under the lack of LH. The pachytene spermatocytes from both immature and adult rats showed vulnerability to undergo apoptosis.

Miething (1992) showed that during prespermatogenesis phase there is a temporal association of prespermatogonial proliferation and degeneration until at least 13th day after birth in the golden hamsters. Such a phase of profound degeneration seems to have slowed down by day 21 in the rats which may also be attributed to the decrease in the relative number of prespermatogonia. Though there are several reports on the spontaneous apoptotic cell death (Allan *et al.*, 1992; Bartke, 1995), the detection of the same may be difficult through DNA fragmentation. One reason may be the rapid phagocytosis of apoptotic cells by the Sertoli cells. It could be possible that the withdrawal of the trophic hormones amplifies the degenerative changes which become detectable through DNA fragmentation. This may be one of the reasons for the DNA fragmentation being not detectable in the fractions from the control and other fractions from LHa/s treated rats.

Although there are many recent reports on the molecular mechanism underlying the process of apoptotic cell death, the effector mechanism is still incompletely known (Boise *et al.*, 1993; Buttyan *et al.*, 1989; Oltvai *et al.*, 1993; Martin *et al.*, 1994; Raff, 1992; Wong *et al.*, 1993; Zhong *et al.*, 1993). The

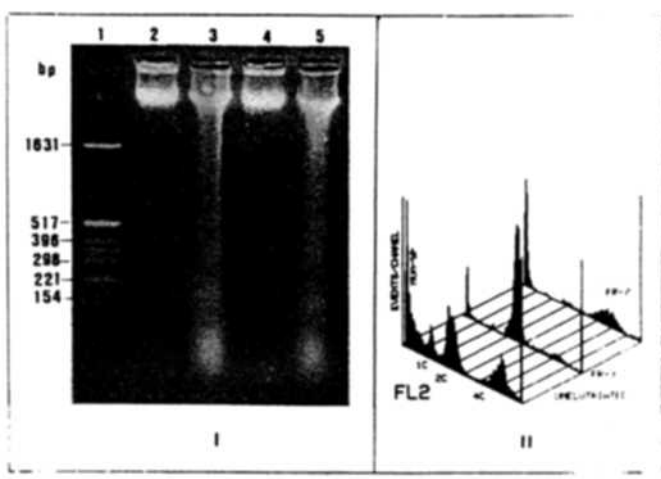

Figure 3 Identification of testicular germ cell types sensitive to lack of LH (Immature rats). (I) Twenty-one day old rats were treated with oLH a/s for 2 days ($n = 35$). The animals were sacrificed 24 h after second injection and the total pooled germ cells were used to purify different cell types. Approximately 8×10^7 to 2.5×10^8 purified cells of each cell type were used for DNA isolation. DNA (30 μg) from each cell type was electrophoresed on 2% agarose gel. Lane 1: pBR Hinf markers; Lane 2: Control, unelutriated; Lane 3: LH a/s, unelutriated; Lane 4: LH a/s, spermatogonia; Lane 5: LH a/s, tetraploid cells. (II) DNA flow cytometric profile of the germ cell population showing the relative purity. The peaks 1C, 2C and 4C respectively represent haploid cells (spermatids), diploid cells (primarily spermatogonia) and tetraploid cells (primarily pachytene spermatocytes). Fr.3: Fraction enriched with spermatogonia; Fr 7: Fraction enriched with tetraploid cells.

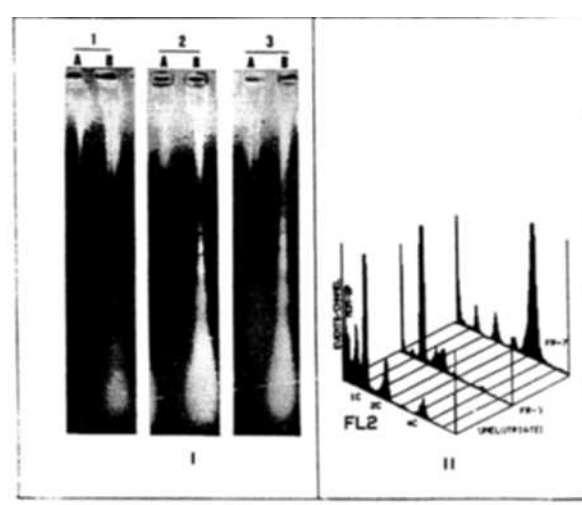

Figure 4 Identification of testicular germ cell types sensitive to lack of LH (Adult rats). (I) Adult rats ($n = 4$) were passively immunized with oLH a/s (300 μl/rat/day) for two days. Twenty-four hours after the second injection, the animals were sacrificed and pooled germ cells were used for purification of each cell type by centrifugal elutriation. Approximately 1×10^8 to 3×10^8 purified cells obtained after elutriation were subjected to DNA isolation and 30 μg DNA was electrophoresed on 2% agarose gel. Lane 1A: Control, unelutriated; Lane 1B: LH a/s, unelutriated; Lane 2A: Control, spermatids; Lane 2B: LH a/s, spermatids; Lane 3A: Control, tetraploid cells; Lane 3B: LH a/s, tetraploid cells. (II) DNA flow cytometric profile of the germ cell population showing the relative purity. The peaks 1C, 2C and 4C represent haploid cells (spermatids), diploid cells (primarily spermatogonia) and tetraploid cells (primarily pachytene spermatocytes) respectively. Fr 3: Fraction enriched with spermatids; Fr 7: Fraction enriched with tetraploid cells.

present experimental model can serve as a reliable model for investigating the expression of some genes during apoptosis. The study along with our earlier observations (Shetty *et al.*, communicated) suggests that the LH is germ cell differentiating as well as a germ cell survival factor and the testosterone mediates the suppression of apoptotic cell death in testicular germ cells. The apoptotic DNA fragmentation can be employed as a useful and sensitive marker in the study of hormonal regulation of spermatogenesis.

Materials and methods

Hormones

Ovine LH used in the study was obtained from Dr G.S. Murthy, Indian Institute of Science and was purified and characterized as described earlier (Moudgal *et al.*, 1992). Rat LH used in the study was obtained from NIADDK.

LH antiserum

Polyclonal antibodies against ovine LH were raised in the rabbits according to the standard protocol (Madhwaraj and Moudgal, 1970). Ability of this antibody to bind and neutralize rat LH was demonstrated by incubating ^{125}I labelled rat LH with this antibody and determining the binding. Further, biological neutralization of rat LH was demonstrated by administering different amounts of the antiserum to adult rats and determining the serum testosterone levels 3 h after the injection. In addition, the antibody was also administered to 21 day immature male rats for 5 to 7 days and the decrease in the testicular weight was determined.

Animals/treatment

Male wistar rats obtained from the Institute's rat colony were used throughout the study. The experiments were carried out with both immature (21 day old weighing approximately 35 g) and adult rats (60 day old weighing approximately 150–200 g). In the first experiment, six immature rats were administered intraperitoneally 75 µl of oLH a/s along with or without 3 mg/day of testosterone with a gap of 24 h in between two injections. The control animals received normal rabbit serum (NRS). The animals were sacrificed 24 h after the first or second injection depending on the experimental schedule. The testes were removed and immediately frozen in liquid nitrogen and stored at − 70°C until further processing for DNA isolation. In the second experiment, 35 21 day old immature rats were administered with 75 µl of oLH a/s per day for 7 days and 24 h after the last injection the animals were sacrificed, testes were decapsulated and the pooled testicular cells were subjected to centrifugal elutriation for purification of different germ cells. Twenty NRS treated rats served as controls. In the third experiment, four adult rats were treated with 200 µl of oLH a/s per day for 2 days. Twenty four hours after the second injection, the animals were sacrificed and the pooled testicular germ cells were used for purification of different germ cells by centrifugal elutriation.

All the experiments were repeated 3 times.

Tissue preparation and purification of different populations of germ cells

The germ cells were purified by centrifugal elutriation according to the procedures of Bucci *et al.* (1986) with several modifications in the rotor speed and the flow rates in order to obtain maximal purification of germ cells (Table 1). Briefly, decapsulated testicular minces were digested with collagenase (0.1% in Dulbecco's minimum essential medium (DMEM) containing 25 mM Hepes and DNAse (25 µg/ml) for 45′ and 32°C in a gyrating water bath (150 cycles/min).

Table 1 Elutriation conditions employed for separation of testicular germ cells from 28 days old rat

Fraction no.	*Flow rate*[3] *ml/min.*	*Cell type enriched*[1,2]
1	7.7 ± 0.8	–
2	10.5 ± 1.2	Cytoplast
3	19.9 ± 1.2	RBC, spermatogonia (40%)
4	24.7 ± 1.2	Spermatogonia (70%)
5	29.5 ± 2.1	Spermatogonia (25%) + round spermatids (43%)
6	42.9 ± 3.7	Round spermatids (77%) + early tetraploid cells (23%)
7	60.5 ± 7.2	Late tetraploid cell (68%)

Testes was removed from 20 immature rats, digested with collagenase and single cell preparation was loaded into Beckman elutriator rotor at 2,650 r.p.m. The flow rate was varied as indicated. The cells from each fraction were stained with hematoxylin and eosin. Cells were also fixed in 70% ethanol for DNA flow cytometric analysis which was carried out according to the procedure of Suresh *et al.* (1993). [1]The number in the parenthesis indicate % of the major population of cells. [2]The cell types were identified by morphology and flow cytometry. [3]Flow rates presented are the mean ± s.d. of three different experiments

Table 2 Elutriation conditions employed for the separation of cells from adult rats

	Rotor speed (r.p.m.)	*Flow rate (ml/min.)*	*Volume (ml)*	*Fraction enriched with*[1,2]
1	3000	12.0	200	Spermatozoa, elongated spermatids
2	3000	16.5	150	RBCs and Cytoplasts
3	3000	30.0	150	Spermatogonia, elongating spermatids
4	2000	23.0	150	Round spermatids (80%)
5	2000	36.0	150	
6	1000	13.0	150	Pachytene spermatocytes (75%)
7	1000	20.0	150	

Testes was removed from 4 adult rats, digested with collagenease and single cell preparation was loaded into Beckman elutriator rotor at 3,000 r.p.m. The flow rate and the rotor speed were varied as indicated. The cells from each fraction were stained with hematoxylin and eosin. Cells were also fixed in 70% ethanol for DNA flow cytometric analysis which was carried out according to the procedure of Suresh *et al.* (1993). [1]The number in the parenthesis indicate % of the major population of cells. [2]The cell types were identifed by morphology and flow cytometry

The cells were centrifuged, washed and resuspended in Ca^{+2} and Mg^{+2} free phosphate buffered saline containing 0.1% glucose and 0.2% bovine serum albumin (BSA) and loaded into an elutriator rotor (Beckman, JE-6). The rotor speed was kept constant at 2650 r.p.m. and the flow rate was varied from approximately 6 ml/min to 40 ml/min and at each speed 150 ml fractions were collected. The cells in each fraction were centrifuged at 100 × g for 7 min, washed once with BSA free buffer and resuspended according to the experimental protocol. For purification of germ cells from the adult rat testis, a different protocol similar to that described by Meistrich *et al.* (1981) with some modifications was used (Table 2).

An aliquot of each cell population was also fixed in 70% ethanol for DNA flow cytometric analysis and quantification of individual cells in different fractions. The germ cell preparation, DNA staining and flow cytometric analysis were carried out according to the procedures standardized by Suresh *et al.* (1992). The flow cytometer used in this study was obtained from Beckton-Dickinson (FAC Scan) and was equipped with an air-cooled 15 milliwat argon-ion laser. The excitation and emission wavelengths were 488 nm and 545 nm respectively. 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 leukocytes as the diploid standard (Suresh *et al.*, 1992).

DNA isolation and gel electrophoresis

Total DNA was isolated from the frozen tissue as described by Sambrook *et al.* (1989) and quantitated by absorbance at 260 nm. DNA (30 μg lane) was electrophoresed on 2% agarose gel for 2 h at 50 volts.

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