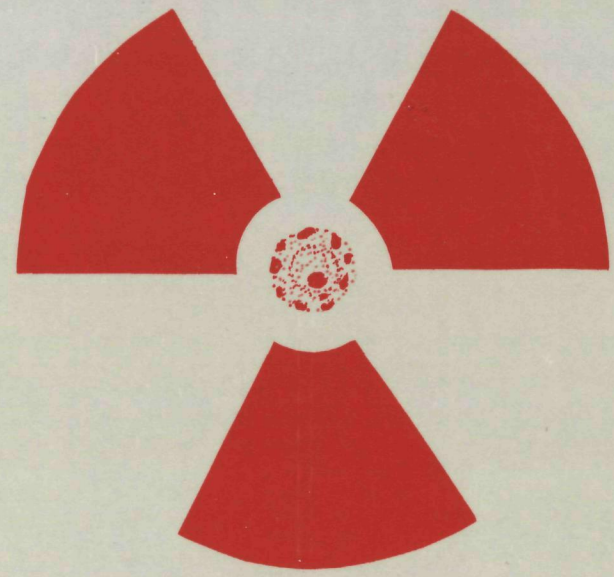
HORMONAL PROTECTION OF SPERMATOGENIC STEM CELLS DURING IRRADIATION

A histological, hormonal and flow cytometric study in rats



M.J.P.G. van Kroonenburgh

HORMONAL PROTECTION OF SPERMATOGENIC STEM CELLS DURING IRRADIATION

HORMONAL PROTECTION OF SPERMATOGENIC STEM CELLS DURING IRRADIATION

A histological, hormonal and flow cytometric study in rats

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. J.H.G.I. GIESBERS
VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 16 OKTOBER 1986
DES NAMIDDAGS TE 3.30 UUR

door

MARINUS JOANNES PETRUS GERARDUS VAN KROONENBURGH

Geboren te Heerlen

krips repro meppel
1986

PROMOTORES

Prof. Dr. W.A.J. van Daal
Prof. Dr. R. Rolland

CO-REFERENTEN

Prof. Dr. C.J. Herman
Dr. H.M. Vemer

The studies presented in this thesis were performed in the Departments of Radiotherapy (Prof. Dr. I. Kazem; at present Prof. Dr. W.A.J. van Daal), Pathology (Prof. Dr. G.P. Vooys), Gyneacology and Obstetrics (Prof. Dr. R. Rolland) and in the Central Animal Laboratory (Dr. W.J.I. van der Gulden), Sint Radboud Hospital and Medical Faculty, Catholic University of Nijmegen, the Netherlands.

Thus the task is not so much to see what no one has seen yet, but to think what nobody has thought yet, about what everybody sees.

Spinoza

CONTENTS

CHAPTER 1

INTRODUCTION

With the increasing success of irradiation and chemotherapy in prolonging the lives of patients with malignant diseases one started to realize that permanent damage to self-renewing cell populations may be induced. Damage to the gonadal cells causes infertility and often permanent sterility (17,21,36,37). Since a large proportion of patients undergoing treatment is young, this particular side-effect may create serious problems. Radiation therapy, for instance in the early stages of Hodgkin disease (16), is leading to prolongation of life in persons of child bearing age. However, gonadal dysfunction occurs (9,19,21,34,35) as an important iatrogenic side effect. In male patients this can result in permanent azoospermia or severe oligospermia (7,8,35). Even when the testes are shielded, internal radiation scatter is still a significant problem (35). This side effect of exposure to radiation is of increasing importance. Current research efforts are made (6,14) to decrease treatment related complications. A treatment which would reduce the magnitude of testicular damage after irradiation is highly desirable and could result in preservation of reproductive function.

Many authors (18,20,22,23,24,25) have reported spermatogonia to be the most radiosensitive germ cells. It was possible to selectively destroy most of the spermatogonial population by irradiation without damaging other cells. Depletion of spermatogonia resulted in disappearance of cells in their order of developmental sequence and eventually resulted in loss of sperm. However, as early as 1955, Oakberg (22,23) remarked on the heterogeneous sensitivity of spermatogonia in the mouse, noting that some survived doses as high as 15.0 Gray (Gy) with eventual return of fertility. This was confirmed in a number of subsequent studies (3,4,27,28,29,30,31,38). The type of spermatogonium which is radiation resistant, is the most important cell type in reference to fertility effects of radiation. The pivotal question concerning the identification and characterization of the radioresistant

spermatogonia was answered by studies (10,13,26) of irradiated testes. The radioresistant spermatogonia appeared to be the isolated stem cells found in normal testis. Shortly after testicular irradiation stem cell spermatogonia are almost the only spermatogonia present (5,12,13). They keep their capacity to replenish their own numbers and to differentiate and eventually contribute to restoration of the spermatogonial population. All long term effects of testicular irradiation are primarily related to the response of these stem cell spermatogonia.

It has also been observed (20,26) that depletion of the spermatogonial population by irradiation provokes enhanced mitotic activity among surviving stem cells throughout the epithelium and a large number of these cells enter the S-phase of their cell cycle at times when they do not normally synthesize DNA (11). Erickson (4) observed in the rat stem cell that mitosis is extremely sensitive to low dose irradiation. It was suggested (2,28,31,32,38), that the sharp increase of stem cell radiosensitivity occuring shortly after irradiation, could be attributed to a change of surviving stem cells towards a much more radiosensitive phase of their cell cycle. The implications of such an effect would suggest a particular vulnerability of the stem cell at particular intervals, a vulnerability that could lead to lasting sterility through elimination of the stem cells themselves. Conflicting results have been reported concerning stem cell survival after fractionated irradiation. Withers et al. (38) and Lu et al. (15) showed enhanced stem cell survival when the dose was split into two equal fractions, whereas Oakberg (28) and Cattanach (1) reported no consistent changes. Some studies (31,32,33) showed split-dose irradiation indeed to decrease the number of surviving stem cells depending on the time interval between the two dose fractions. In this way the surviving spermatogonial stem cells play a critical and fundamental role in assuring the perpetuation of the germ line. The triggering factor, however, provoking altered stem cell kinetics in response to irradiation remains unknown. Even the mechanisms involved in inducing some stem spermatogonia to enter the proliferating compartment under normal physiological circumstances still has to be solved.

It seems reasonable to suppose that the germinal epithelium is stimulated to compensate for a failure in maintaining normal spermatogenesis. Surviving stem cells might be stimulated after irradiation to divide and thus be at increased risk for further damage. Because spermatogenesis is a highly regulated, hormonally controlled proliferative system, our hypothesis is that inhibition of spermatogenesis by hormones may reduce the stimulus of stem cells to move into the very radiosensitive phase. Inhibition of follicle stimulating hormone (FSH) and/or luteinizing hormone (LH) release or inhibition of their effects on the testicle could place the testis in a state comparable to that of prepuberty. This resting state could make the germinal epithelium more resistant to irradiation and could in this way protect the stem cell from depletion. To test this hypothesis a rat model was investigated where irradiation produced severe damage to spermatogonia and administration of various hormonal substances was used to achieve reversible inhibition of spermatogenesis.

References

1. Cattanach BM: Spermatogonial stem cell killing in the mouse following single and fractionated X-ray doses, as assessed by length of sterile period. Mut. Res. 25, 53-62, 1974.

2. Clow DJ and Gillette EL: Survival of type A spermatogonia following X-irradiation. Radiat. Res. 42, 397-404, 1970.

3. Dym M and Clermont Y: Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. Am. J. Anat. 128, 265-282, 1970.

4. Erickson BH: Effect of Co^{60} γ radiation on the stem and differentiating spermatogonia of the post pubertal rat. Radiat. Res. 68, 433-448, 1976.

5. Erickson BH: Survival and renewal of murine stem spermatogonia following Co^{60} γ radiation. Radiat. Res. 86, 34-51, 1981.

6. Glode LM, Robinson J and Gould SF: Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropin-releasing hormone. Lancet, 1132-1134, 1981.

7. Greiner R: Die Erholung der Spermatogenese nach fraktionierter, niedrig dosierter Bestrahlung der mannlichen Gonaden. Strahlentherapie. 158, 342-355, 1982.

8. Hahn EW, Feingold SM and Nisce L: Aspermia and recovery of spermatogenesis in cancer patients following incidental gonadal irradiation during treatment. Radiology 119, 223-225, 1976.

9. Horning SJ, Hoppe RT, Kaplan HS and Rosenberg SA: Female reproductive potential after treatment for Hodgkin's disease. N. Eng. J. Med. 304, 1377-1382, 1981.

10. Huckins C: The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat. Rec. 169, 533-558, 1971.

11. Huckins C: Behavior of stem cell spermatogonia in the adult rat irradiated testis. Biol. Reprod. 19, 747-760, 1978.

12. Huckins C: Spermatogonial intercellular bridges in whole mounted seminiferous tubules from normal and irradiated rodent testes. Am. J. Anat. 153, 97-122, 1978.

13. Huckins C and Oakberg EF: Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II. The irradiated testes. Anat. Rec. 192, 529-537, 1978.

14. Johnson DH, Linde R, Hainsworth JD, Vale W, Rivier J, Stein R, Flexner J, Van Welch R and Greco FA: Effect of a luteinizing hormone releasing hormone agonist given during combination chemotherapy on posttherapy fertility in male patients with lymphoma: Preliminary observations. Blood 65, 832-836, 1985.

15. Lu CC, Meistrich ML and Thames HD: Survival of mouse testicular stem cells after γ or neutron irradiation. Radiat. Res. 81, 402-415, 1980.

16. Lushbaugh CC and Casarett GW: The effects of gonadal irradiation in clinical radiation therapy: A review. Cancer 37, 1111-1120, 1976.

17. Matus-Ridley M, Nicosia SV and Meadows AT: Gonadal effects of cancer therapy in boys. Cancer 55, 2353-2363, 1985.

18. Meistrich ML, Hunter NR, Suzuki N, Trostle PK and Withers HR: Gradual regeneration of mouse testicular stem cells after exposure to ionizing radiation. Radiat. Res. 74, 349-362, 1978.

19. Mercke CE, Yabe I, Landberg TG and Svahn-Tapper GO: Hodgkin's disease irradiated with the inverted-Y technique. Acta Rad. Oncol. 20, 81-89, 1981.

20. Monesi V: Relation between X-ray sensitivity and stages of the cell cycle in spermatogonia of the mouse. Radiat. Res. 17, 809-838, 1962.

21. Nicosia SV, Matus-Ridley M and Meadows AT: Gonadal effects of cancer therapy in girls. Cancer 55, 2364-2372, 1985.

22. Oakberg EF: Sensitivity and time of degeneration of spermatogenic cells irradiated in various stages of maturation in the mouse. Radiat. Res. 2, 369-391, 1955.

23. Oakberg EF: Degeneration of spermatogonia of the mouse following exposure to X-rays and stages in the mitotic cycle at which cell death occurs. J. Morph. 97, 39-54, 1955.

24. Oakberg EF: Initial depletion and subsequent recovery of spermatogonia of the mouse after 20 R of γ rays and 100, 300, and 600 R of X-rays. Radiat. Res. 11, 700-719, 1959.

25. Oakberg EF: The effects of dose rate and quality of radiation on the dynamics and survival of the spermatogonial population of the mouse. Jap. J. Genet. (Suppl.) 40, 119-127, 1964.

26. Oakberg EF: Spermatogonial stem cell renewal in the mouse. Anat. Rec. 169, 515-532, 1971.

27. Oakberg EF: Effects of radiation on the testis. In Handbook of Physiology Section 7. Endocrinology Vol. V (ed. Greep RO et al. Am. Phys. Soc. Washington), 233-243, 1975.

28. Oakberg EF: Differential spermatogonial stem cell survival and mutation frequency. Mut. Res. 50, 327-340, 1978.

29. Oakberg EF and Huckins C: Spermatogonial stem cell renewal in the mouse as revealed by H^3-thymidine labeling and irradiation. J. Endocr. 60, 287-293, 1974.

30. Partington M, Craig AW and Jackson H: The effect of radiation on spermatogenic cells in the rat and mouse. Br. J. Radiol. 35, 713-718, 1962.

31. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean energy or 300 KV X-rays. Radiat. Res. 71, 579-592, 1977.

32. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean energy. Effect of the fractionation interval. Radiat. Res. 79, 289-297, 1979.

33. Sheridan W: The effects of the time interval in fractionated X-ray treatment of mouse spermatogonia. Mut. Res. 13, 163-169, 1971.

34. Specht L, Geisler C, Hansen MM and Skakkebaek NE: Testicular function in young men in long-term remission after treatment for the early stages of Hodgkin's disease. Scand. J. Haem. 33, 356-362, 1984.

35. Speiser B, Rubin P and Casarett G: Aspermia following lower truncal irradiation in Hodgkin's disease. Cancer 32, 692-698, 1973.

36. Viviani S, Santoro A, Ragni G, Bonfante V, Bestetti O and Bonadonna G: Gonadal toxicity after combination chemotherapy for Hodgkin's disease. Comparative results of MOPP vs ABVD. Eur. J. Canc. Clin. Oncol. 21, 601-605, 1985.

37. Whitehead E, Shalet SM, Blachledge G, Todd I, Crowther D and Beardwell CG: The effects of Hodgkin's disease and combination chemotherapy on gonadal function in the adult male. Cancer 49, 418-422, 1982.

38. Withers HR, Hunter N, Barkley HT and Reid BO: Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testes. Radiat. Res. 57, 88-103, 1974.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Spermatogenesis of the rat

The seminiferous epithelium of adult, sexually mature rats is composed of differentiating germ cells intercalated between the supporting Sertoli cells. The mitotically active cells in the seminiferous epithelium are called spermatogonia. These cells are found exclusively on the basement membrane of the seminiferous tubules below the sertoli junctional complexes. After a series of divisions, spermatogonia form primary spermatocytes, which grow, leave the basement membrane and move towards the lumen. During this period of growth the nucleus of the primary spermatocyte undergoes a series of changes before the first meiotic division appears. After full development the primary spermatocyte divides into two smaller secondary spermatocytes, (second meiotic division) by which spermatids containing a haploid number of chromosomes are produced. Coincident with the onset of meiosis, the early primary spermatocytes transverse the occluding sertoli junctions. As differentiation proceeds, the spermatocytes and early spermatids are gradually moved towards the lumen of the seminiferous tubule. Spermatids become closely connected with the Sertoli cell and undergo an extraordinarily complex series of changes, leading without further divisions to the production of the highly differentiated germ cell, the spermatozoon (23).

The spermatogonia, spermatocytes and spermatids at various steps of development do not occur at random but form well-defined cellular associations. Analysis of the composition of these various cell associations reveals that they appear in a given area of a tubule and follow each other in a given and fixed sequence. Since such a sequence must repeat itself, the seminiferous epithelium as a whole evolves in a cyclic manner and a complete series of typical cell associations constitutes "a cycle of spermatogenesis". The typical cell associations are referred to as stages of the cycle. Such

stages represent artificial subdivisions through a continous process and vary depending on the criteria to characterize or identify them (9).

With the advent of radioactive tracers and the development of autoradiography it became possible to determine the duration of the spermatogenic "cycle". Tritiated thymidine, which is selectively incorporated in the nucleus of cells has been used extensively to time the cycle. Huckins (38) determined the duration of the cycle in the wistar rat to be 13.3 days, whereas Hilscher (36) estimated a duration of 13.0 days. This cycle length seems to be constant. The duration of the whole process of spermatogenesis was estimated to be 53.2 and 53.0 days respectively. In 1968 Clermont and Bustos-Obregon (10) introduced an alternative technique for studying spermatogonia using whole mounts of seminiferous tubules instead of sections. This led to a more detailed insight into the different types of spermatogonia. Their study presented evidence for the existence of two main classes of type A spermatogonia stem cells. They observed an "isolated" germ cell in the rat testis that did not appear to participate regularly in the spermatogenic cycle. Since mitoses of these cells were seen infrequently, the authors proposed that these spermatogonia represented reserve stem cells, which did not normally participate in the spermatogonial stem cell renewal. This cell type was named consequently as "Ao" stem cell (Fig. 2.1). The other cells, which participated in the spermatogenesis were referred to as renewing stem cells.

During the past decade most of the research has been focussed on the spermatogonia. Huckins (39) and Oakberg (67) further scrutinized the "Ao" spermatogonium by means of isotope and morphological techniques and came to the conclusion that spermatogonial stem cells came from a continously cycling population and not from a non-cycling one as required by the "Ao" concept. The stem cell was probably not a reserve stem cell. The stem cell should therefore be designated as "As" (s for stem). It was suggested that these "As" cells occur isolated rather than in groups and are distributed randomly throughout the length of the seminiferous tubule. Huckins (39) proposed a new hypothesis for a stem cell renewal model in the rat on the basis of quantitative reevaluation of whole mounts of

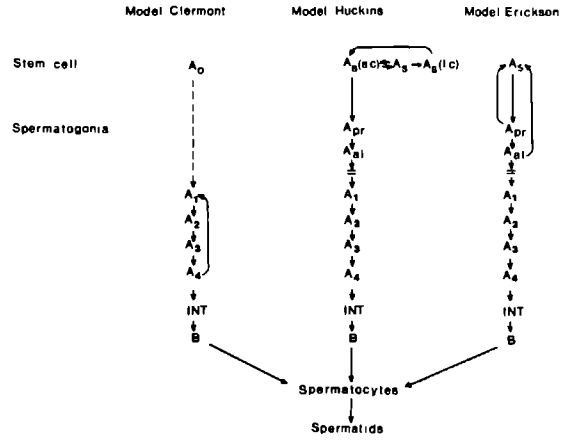


FIG. (2.1) *Diagrammatic representation of spermatogonial stem cell renewal for the rat as proposed by Clermont and Bustos-Obregon (10), Huckins (40) and Erickson (22).*

seminiferous tubules. In this model (Fig. 2.1), the daughter cells of the "As" cells retain their proximity and form pairs of spermatogonia (Apr) or they can divide to form more single "As" cells (self-renewal). Successive synchronous divisions of the pair lead to the formation of a chain of 4, 8 or 16 A aligned spermatogonia (Aal), which retain the "As" nuclear morphology. However, these "Aal" cells stop dividing and transform into A1 spermatogonia. Pair formation is considered to be the initial step in an irreversible developmental sequence. The chain of "Aal" cells appear to have contiguous cytoplasm under the light microscope (44) and with the electron microscope intercellular bridges were observed (18) between spermatogonia. These bridges are an important factor in maintaining the synchrony in spermatogonial proliferation. All members of a given chain are morphologically identical and are in the same phase of their mitotic cycle.

Intriguingly more and more evidence arose that this stem cell

compartment "As" is probably composed of more classes (34). Autoradiographical (40,67), and radiobiological studies (6,14,50,68,77,97) show indications for heterogenic behaviour of spermatogonial "As" stem cells. Wether these subpopulations represent two different cell types (6,50) or different stages in the cell cycle of a single cell (14,68,77,97) still remains to be answered.

2.2 Regulation of spermatogenesis

Germinal epithelial cells proliferate continously throughout the adult life of a male requiring a constant supply of germ cell precursors. Large groups of germ cells evolve synchronously throughout the spermatogenic process. Spermatogonia must arise periodically to replace those that differentiate into spermatocytes, which in turn yield spermatids and spermatozoa. The mechanisms responsible for regulating this process have been the topic of many investigations, and, although a number of theories have been proposed, including those that Sertoli cells are transformed into germinal cells or that cells of the wall of the seminiferous epithelium may transform into spermatogonia, evidence points towards the principle of a self-renewing germinal stem cell.

2.2.1 Stem cell renewal

The precise manner in which stem cells renew remains poorly understood, although there are some theories (Fig. 2.1):

1. Clermont and Bustos-Obregon (10) divide the spermatogonia of the rat into two groups. The first contributes the actively renewing population and comprises the cell types A1-A4 spermatogonia. The second group consists of a cell type designated as "Ao", which are non-dividing single cells. These cells are considered to be reserve stem cells that become active only if the more mature spermatogonial types are depleted by a noxious agent such as radiation.

2. In the stem cell renewal model of Huckins (40) the stem cell "As" has two potentials: It divides to form more single "As" spermatogonia; or division may result in the formation of a pair of cells (Apr). Pair formation is considered to be the initial step in an irreversible sequence.

3. According to Erickson (22), the stem cell (As) could possibly be produced by breakage of intercellular bridges between A paired (Ap) and A aligned (Aal) cells. Thus "Ap" and "Aal" cells might split into two "As" spermatogonia.

Evidence from experimental studies (21,45,49,67,69,76,97) in which the spermatogonial population was depleted, clearly fits best with the stem cell renewal model of Huckins (40).

2.2.2 Regulation of spermatogonial proliferation

The complexitiy of producing vast numbers of germ cells in a precise manner and coordinative pattern requires regulatory mechanisms. The mechanisms which regulate this process or the factors, which are responsible for this regulation still remain unclarified. Hormones certainly are involved in the regulation of spermatogonial proliferation (see chapter 2.2.3).

Because spermatogenesis is a very rigid time scaled process it was suggested that some intratesticular mechanisms must exist to regulate this proliferation. The depletion of differentiating germ cells from the testis following X-irradiation leads to an apparent compensatory increase in the proliferation of the spermatogonial stem cells (17,41,43). Huckins and Oakberg (45) noticed that enhanced activity of stem cells coincided with the period of the epithelial cycle in which many differentiating spermatogonia degenerate, suggesting a local feedback mechanism. It was also observed that the cycle of the seminiferous epithelium proceeded more rapidly in juvenile than in adult animals (38). Another phenomenon which should be mentioned here is the observation of van Keulen and de Rooij (48,49) that after severe cell loss the production of spermatogonia type A paired is inhibited for a certain

time. These observations suggest that the proliferation of spermatogonia is modulated by some intragonadal factors.

Most locally acting factors in a cell renewing system generally are small peptides. A survey of the current literature (82) shows a substantial list of potential factors, such as oxytocin, vasopressin, endorphins, thyreotropin releasing hormone, epidermal growth factor and somatodins, which may be involved in local regulation of testicular function. It was also suggested that spermatogonial proliferation is subject to feedback regulation via a chalone-like substance. Several authors (12,46,92) found proliferation of spermatogonia in the rat to be inhibited by injection of a testicular extract. As yet this "chalone" has not been isolated and it is still unknown which cell is responsible for its production. In which respect Sertoli cells influence spermatogonial proliferation, directly or indirectly, still remains to be clarified. Feig et al. (24) claimed to have found a mitogenic polypeptide. This mitogenic protein, the seminiferous growth factor, is presumed to play a fundamental role in testicular physiology (2). Recently it was proposed by Sharpe (82) that an LH-RH like peptide is produced by the Sertoli cells within the testis, acting specifically in the Leydig cells interacting with LH and hence modulating the production of testosterone. As yet it is not certain how local factors play a role in the regulation of spermatogonial proliferation.

2.2.3 Hormonal control of spermatogenesis

It has been clearly established in man, as in other mammalian species, that normal spermatogenesis requires the stimulatory action of pituitary gonadotropins (FSH and LH). The classical study of Smith (85) in 1930 unequivocally demonstrated pituitary control of gonadal functions in the rat and thus established firmly the importance of gonadotropins in testicular physiology. Clermont and Morgenthaler (13) showed in a quantitative morphological study in rats an extensive degeneration of germinal cells with a decreased number of spermatogonia and early spermatocytes following surgical removal of the pituitary. Also the numbers of pachytene

spermatocytes and spermatids drastically decreased, specifically at stage VII of the cycle of the seminiferous epithelium. Soon after partial purification of gonadotropins, the concept was proposed that LH acted on the Leydig cells in the interstitium, stimulating testosterone production, whereas FSH controlled spermatogenesis (27,28). It was demonstrated (15) that LH binds specifically to Leydig cells, where it stimulates cyclic AMP accumulation and the conversion of cholesterol to pregnenolone at the mitochondrial level, leading to increased formation of testosterone. The roles of FSH and testosterone in the regulation of spermatogenesis have been the subject of controversy for many years. The observations, that exogenous androgens can also maintain spermatogenesis in hypohysectomized rats (61,95) confused the originally proposed neat design (27) of FSH control in the regulation of spermatogenesis.

Many studies were set up to learn which part of germ cell development was influenced by the different hormones. In 1971, it was generally assumed that the various stages of spermatogenesis require specific but not necessarily the same hormones (87). FSH was thought to act on the completion of spermiogenesis and formation of spermatozoa, whereas completion of the meiotic division was described (88) to be a specific testosterone-dependent step in spermatogenesis (87,88). In the last ten years more and more evidence, however, suggests that the effects of FSH and testosterone are mediated by the Sertoli cells (30,31). It seems that Sertoli cells are the primary site of FSH action in the testis and that production of a specific androgen binding protein (ABP) by the Sertoli cells is stimulated by FSH (89). Direct binding of FSH to tubules containing only Sertoli cells is in agreement with this (32). Binding of FSH to Sertoli cells is followed by cyclic-AMP accumulation, protein kinase activation, RNA synthesis and ABP production (55). Rat Sertoli cells in culture secrete a Sertoli cell factor (SCF) which selectively suppresses FSH production by pituitary cells in vitro (91). Such a substance was first postulated in 1932 by McCullagh (54). This substance was further investigated under the name inhibin (33,52,90). More data must be derived to explain the exact role of an inhibin substance in the feedback regulation exerted in the pituitary gland. Secretion of ABP by

Sertoli cells was shown to be correlated with the state of spermatogenesis under different hormonal conditions (74). There is evidence that the Sertoli cells are also target cells for androgens (29,60,94). Studies using immature or adult hypophysectomized rats, revealed that testosterone can stimulate the activity of ABP as can FSH (20,80,93,96). At the moment it is generally assumed that the effects of FSH and testosterone on spermatogenesis are mediated by Sertoli cells. Still the mechanism by which Sertoli cells act upon germ cells and especially on spermatogonial proliferation remains to be elucidated.

A direct influence of FSH and testosterone on spermatogonial proliferation has not been well established to date, although FSH binding to spermatogonia was suggested by Orth and Christensen (70). Chowdhury (8) showed that spermatogonia may be partially dependent in their function on testosterone-propionate and/or gonadotropins. FSH seems to be particularly important for initiation of spermatogenesis during the development of the testis in immature rats (51) and also during the development as well as for restoration of spermatogenesis after gonadal damage (19,87). However, the effects of FSH and testosterone on spermatogonia are peculiar in that they can not be demonstrated in intact adult rats. Such data raised the assumption that FSH and/or testosterone may be important only during the critical period of initiation of spermatogenesis (25). It is possible that FSH and/or testosterone in this way are essential in stimulating stem cells after severe loss to produce new spermatogonia. This role of FSH would be compatible with the apparent loss of spermatogonia in prepubertal rats following treatment with an FSH antibody (7).

2.3 Testicular irradiation

Soon after the discovery of X-rays it was realized that these penetrating radiations induced striking alterations in living tissue. In 1903 Albers-Schönberg (1) first described radiation damage to the testis. In 1905 Bergonie and Tribondeau (3) noticed the skin of the scrotum to be unaffected despite the fact that the animal lost its fertility. Régaud and Blanc (75) recognized among the germ cells of the testis, spermatogonia to be extremely sensitive to X-rays. On the basis of early experimental studies of radiation effect on the testes, Bergonie and Tribondeau (4) postulated that the radiosensitivity of cells is determined by their degree of differentiation, their mitotic rate and their mitotic future. Experimental evidence later on (35,53,81) confirmed that spermatogonia with a high mitotic rate were much more sensitive than spermatocytes or spermatids. This high radiosensitivity of spermatogonia, however, could also be related to the presence of intercellular bridges (42), which play an important role in the synchrony of an entire chain of proliferating spermatogonia: An entire chain dies or survives. Depletion of spermatogonia resulted in disappearance of cells in their order of developmental sequence and eventually resulted in loss of sperm. However, a few spermatogonia survived and could repopulate the germinal epithelium. Thus both the initial depletion and the subsequent regeneration of the spermatogenic elements depend upon the response of the spermatogonia. Later studies on the effect of radiation on mammalian seminiferous epithelium focussed in particular on the spermatogonial population. Testicular irradiation investigations, however, were hampered by either incomplete or inaccurate knowledge of the kinetics of spermatogenesis.

A new impulse in this research came after the Second World War when a better understanding of the effects of irradiation of the testis became possible by an increase in the knowledge of the dynamics and timing of spermatogenesis and especially of the process of spermatogonial cell renewal. Studies on mouse (58,59,62,63,64,65) and rat (5,14,16,36,47,71,83) testes clearly demonstrated that the spermatogonia (intermediate and type B) are particularly sensitive

to irradiation and that type A spermatogonia appear to be of a heterogeneous population. Among type A spermatogonia some are very resistant to irradiation and these surviving cells contribute to restoration of the spermatogonial proliferation.

Questions arose about the identification and characterization of the radioresistant spermatogonia. Utilizing a tubule whole-mount method Clermont and Bustus-Obregon (10) observed in 1968 an isolated germ cell type in rat testis that was not regularly present in the spermatogenic cycle; this cell was called type "Ao" spermatogonia (see chapter 2.1). Dym and Clermont (17) concluded that these stem cells "Ao" were comparitively more radioresistant than spermatogonia type A1. These radioresistant cells were considered to belong to a dormant reserve stem cell population, which became mitotically active only in response to germ cell depletion. Another phenomenon described by them was that the radioresistant spermatogonia entered into spermatogenesis at fixed and predetermined times. In other words, the proliferative activity of radioresistant spermatogonia appears to be a rigidly regulated process. Later on, experimental evidence accumulated showing that stem cells labeled with tritiated thymidine prior to irradiation survived the radiation insult (67), and hence must be in active cell cycle. Huckins (39), Oakberg (67) and Rooij et al. (76) postulated that the isolated spermatogonia are functional stem cells. Moreover it became clear from studies (21,44,45,69) of irradiated testis and observations on whole mounted seminiferous tubules that the radiosurvivers are the cycling type A stem cells found in normal testes.

The stem cell response during irradiation is of great importance in terms of long term radiation effects. Proliferating systems are normally compartmentalized and the cell cycle may be divided into four phases (37): G_1 (the presynthetic period), S (the DNA synthetic period), G_2 (the post synthetic period) and M (the period of mitosis). Radiation profoundly disturbs the kinetics of cellular proliferation and the response of cell renewal systems in general is to establish a new steady state of cell production. The proliferative compartment may expand after cell loss by compensatory cell proliferation, either by increasing the proliferation rate of dividing cells (shortening of cell cycle) or by bringing into

proliferation potentially proliferative non-dividing cells. Huckins (39) and Oakberg (67) noticed that depletion of the spermatogonial population by irradiation provoked enhanced mitotic activity among the surviving "As" stem cells. An increased frequency of cells labeled by tritiated thymidine in the irradiated testis was observed (66). A large number of "As" survivors enter the S-phase of the cell cycle at times when they do not normally synthesize DNA (39). Which factor triggers these cells to enter the S-phase is unknown. Erickson (21,22) described the high radiosensitivity of stem cell mitosis after a second dose of irradiation. Stem cells would be more vulnerable after irradiation because they move into mitosis. This suggested a particular vulnerability of the stem cell at particular intervals. It was shown in the mouse (78) that after a single dose of irradiation stem cells immediately (after 16 hours) became vulnerable. A second dose would destroy these cells and could lead to elimination of the stem cells themselves.

This elimination of stem cells is controversial: Withers et al (97) and Lu et al. (50) showed enhanced stem cell survival when the dose was split into two equal fractions. Ruiter-Bootsma et al. (78,79) and Sheridan (84) demonstrated lower stem cell survival upon fractionation, whereas Oakberg (68) and Cattanach (6) reported no consistent changes in stem cell survival after fractionation. This controversy may be explained by the different methods (50) used in evaluating stem cell survival. Spermatogonial stem cell survival after irradiation injury was studied in mice by histological counts of surviving stem cells (68), counts of repopulated tubular cross sections (78,79,97), sperm head counts (50), LDH-X enzyme activity (50) or by the duration of the sterile period (6,50,84).

Another point of disagreement is the existence of radiosensitive and radioresistant subpopulations of stem cells. Cattanach (6) and Lu et al. (50) found evidence for that existence, while Oakberg (68), Ruiter-Bootsma et al. (78) and Withers et al. (97) did not. Autoradiographic studies (11,21,36) showed that in normal rats, a few of the tritium labeled "As" cells persisted for many days, up to 13 days or more in the seminiferous epithelium. These cells were designated by Huckins (40,41) as long cycling "As" spermatogonia (A1c). Huckins provided evidence for the existence of two

kinetically identifiable populations of "As" (Fig. 2.1) cells: One with a long cycle (Alc) and one with a short cycle (Asc). In case of testicular damage the "Alc" stem cell population could be immediately triggered in short cycling cells. Other authors have reported similar findings (11,21,36,66) but Huckins (41) showed that the radioresistant stem cell has a labelling pattern which is identical to that of the long cycling "As" spermatogonia in normal testis. Huckins suggested that these long cycle stem cells probably are arrested in the presynthetic period of the cell cycle (G_1-phase), like somatic cells. This could explain the high radioresistance of these cells. This possibility was also suggested by Smith and Martin (86). They hypothesized that there is only one stem cell compartment in which the cells cycle at random. Furthermore the heterogeneity in autoradiographic (63) or radiobiologic (50,77,97) behaviour of stem cells can be explained by the different characteristics of the phases of their cell cycle. In this model the stem cells do not constitute two separate compartments but belong to the same population.

2.4 Protection of stem cells during irradiation

A variety of chemical substances have been demonstrated to protect stem cells to some extent from radiation injury (73). A protecting compound against ionizing irradiation was first described by Patt et al. (72) in 1949. A number of large synthesis programs were undertaken in an attempt to find highly effective compounds. They came up with a number of promising compounds, many of which were in the thiophosphate class (98). One of them S-2-(3-aminopropylamino) ethylphosporothioic acid (WR-2721) was shown (56,57) to be able to protect testicular stem cells in mice against irradiation. A synthetic analogue of gonadotropin releasing-hormone was described to ameliorate the gonadal toxicity of systemic chemotherapy (26). No literature, however, is available about the use of hormones in protecting testicular stem cells against irradiation.

References

1. Albers-Schönberg HE: Ueber eine bisher unbekannte Wirkung der Röntgenstrahlen auf den Organismus der Tiere. Münch. Med. Wochenschr. 50, 1859-1860, 1903.

2. Bellvé AR and Feig LA: Cell proliferation in the mammalian testis: Biology of the seminiferous growth factor (SGF) in: Recent progress in Hormone Research 40. ed. Acad. Press Inc. 531-567, 1984.

3. Bergonie J and Tribondeau L: Aspermatogenese experimentale après une seule exposition aux rayons-x. Compt. Rend. Soc. Biol. 58, 282-284, 1905.

4. Bergonie J and Tribondeau L: Interpretation de quelques resultats de la radiotherapie et essai de fixation d'une technique rationelle. Compt. Rend. Acad. Sci. Paris. 143, 983-985, 1906.

5. Casarett AP and Casarett GW: Histological investigations of mechanisms of ray effects on spermatogenesis in the rat. University of Rochester Atomic Energy Project Report UR 496, UR 497, 1957.

6. Cattanach BM: Spermatogonial stem cell killing in the mouse following single and fractionated x-ray doses, as assessed by length of sterile period. Mut. Res. 25, 53-62, 1974.

7. Chemes HE, Dym M and Ray HGM: The role of gonadotropins and testosterone on initiation of spermatogenesis in the immature rat. Biol. Reprod. 21, 241-249, 1979.

8. Chowdhury AK: Dependence of testicular germ cells on hormones: A quantitative study in hypophysectomized testosterone-treated rats. J. Endocr. 82, 331-340, 1979.

9. Clermont Y: Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. Phys. Rev. 52, 198-236, 1972.

10. Clermont Y and Bustos-Obregon E: Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto". Am. J. Anat. 122, 237-248, 1968.

11. Clermont Y and Hermo L: Spermatogonial stem cells in the albino rat. Am. J. Anat. 142, 159-176, 1975.

12. Clermont Y and Mauger A: Existence of a spermatogonial chalone in the rat testis. Cell Tiss. Kinet. 7, 165-172, 1974.

13. Clermont Y and Morgenthaler H: Quantitative study of spermatogenesis in the hypophysectomized rat. Endocrinology. 57, 369-382, 1955.

14. Clow DJ and Gillette EL: Survival of type A spermatogonia following X-irradiation. Radiat. Res. 42, 397-404, 1970.

15. Dufeau ML, Tsuruhara T, Horner KA, Podesta EJ and Catt KJ: Intermediate role of adenosine 3':5'-cyclic monophosphate and protein kinase during gonadotropin induced steroidogenesis in testicular interstitial cells. Proc. Natl. Acad. Sci. USA. 74, 3419-3423, 1977.

16. Dym M and Clermont Y: Effects of x-rays on type A spermatogonia in the rat. Anat. Rec. 157, 238 (Abstract), 1967.

17. Dym M and Clermont Y: Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. Am. J. Anat. 128, 265-282, 1970.

18. Dym M and Fawcett DW: Spermatogonial intercellular bridges. Anat. Rec. 169, 309 (Abstract), 1971.

19. Dym M and Madhwa Raj HG: Response of adult rat Sertoli cells and Leydig cells to depletion of luteinizing hormone and testosterone. Biol. Reprod. 17, 676-696, 1977.

20. Elkington JSH, Sanborn BM and Steinberger E: The effect of testosterone propionate on the concentration of testicular and epididymal androgen binding activity in the hypophysectomized rat. Mol. Cell. Endocrinol. 2, 157-170, 1975.

21. Erickson BH: Effect of Co^{60} γ radiation on the stem and differentiating spermatogonia of the post pubertal rat. Radiat. Res. 68, 433-448, 1976.

22. Erickson BH: Survival and renewal of murine stem spermatogonia following Co^{60} γ radiation. Radiat. Res. 86, 34-51, 1981.

23. Fawcett DW, Neaves WB and Flores MN: Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. Biol. Reprod. 9, 500-532, 1973.

24. Feig LA, Bellvé AR, Erickson NH and Klagsbrun M: Sertoli cells contain a mitogenic polypeptide. Proc. Natl. Acad. Sci. USA. 77, 4774-4778, 1980.

25. Fritz IB: Sites of action of androgens and follicle stimulating hormone on cells of the seminiferous tubule. In Biochemical actions of hormones, Vol.5 (ed. Litwack G) Acad. Press, New York, 249-281, 1978.

26. Glode LM, Robinson J and Gould SF: Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropin-releasing hormone. Lancet. I, 1132-1134, 1981.

27. Greep RO, Fevold HL and Hisaw FL: Effects of two hypophyseal gonadotropic hormones on the reproductive system of the male rat. Anat. Rec. 65, 261-271, 1935.

28. Greep RO and Fevold HL: The spermatogenic and secretory function of the gonads of hypophysectomized adult rats treated with pituitary FSH and LH. Endocrinology. 21, 611-618, 1937.

29. Grootegoed JA, Peters MJ, Mulder E, Rommerts FFG and van der Molen HJ: Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. Molec. Cell. Endocr. 9, 159-167, 1977.

30. Hansson V, Djoseland O, Torgersen O, Ritzen EM, French FS and Nayfeh SN: Hormones and hormonal target cells in the testis. Andrologia. 8, 195-202, 1976.

31. Hansson V, Ritzen EM, French FS and Nayfeh SN: Androgen transport and receptor mechanisms in the testis. In Handbook of Physiology section 7 (ed. Hamilton DW and Greep RO) Vol. 5, 173-201, 1975.

32. Heindel JJ, Rothenberg R, Robinson GA and Steinberger A: LH and FSH stimulation of cyclic AMP in specific cell types isolated from the testis. J. Cyc. Nucl. Res. 1, 69-79, 1975.

33. Hermans WP, van Leeuwen ECM, Debets MHM and de Jong FH: Involvement of inhibin in the regulation of follicle stimulating hormone concentrations in prepubertal and adult, male and female rats. J. Endocr. 86, 79-92, 1980.

34. Hermo L: The existence of two classes of spermatogonial stem cells in the rat testis. Anat. Rec. 175, (Abstr.) 343, 1973.

35. Hertwig P: Die Regeneration des Samenepithels der Maus nach Röntgenbestrahlung unter besonderer Berücksichtigung der Spermatogonien. Arch. Exptl. Zellforsch. Gewebezücht 22, 68-73, 1938.

36. Hilscher W: Beitrage zur Orthologie und Pathologie der "Spermatogoniogenese" der Ratte. Beitr. Path. Anat. 130, 69-132, 1964.

37. Howard A and Pelc SR: Synthesis of desoxy-ribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity (Suppl.) 6, 261-273, 1953.

38. Huckins C: Duration of spermatogenesis in pre-and post puberal Wistar rat. Anat. Rec. 151 (Abstract), 364, 1965.

39. Huckins C: The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat. Rec. 169, 533-558, 1971.

40. Huckins C: The spermatogonial stem cell population in adult rats. III. Evidence for a long cycling population. Cell Tiss. Kinet. 4, 335-349, 1971.

41. Huckins C: Behavior of stem cell spermatogonia in the adult rat irradiated testis. Biol. Reprod. 19, 747-760, 1978.

42. Huckins C: Spermatogonial intercellular bridges in whole mounted seminiferous tubules from normal and irradiated rodent testes. Am. J. Anat. 153, 97-122, 1978.

43. Huckins C and Cunningham GR: Behaviour of spermatogonia in hypophysectomized adult rats. Anat. Rec. (Abstract) 181, 380, 1975.

44. Huckins C and Oakberg EF: Cytoplasmic connections between spermatogonia seen in whole mounted seminiferous tubules from normal and irradiated mouse testes. Anat. Rec. 169 (Abstract), 344, 1971.

45. Huckins C and Oakberg EF: Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II. The irradiated testes. Anat. Rec. 192, 529-537, 1978.

46. Irons MI and Clermont Y: Spermatogonial chalone(s): Effect on the phases of the cell cycles of type A spermatogonia in the rat. Cell Tiss. Kinet. 12, 425-433, 1979.

47. Jones EA: Number of spermatogonia after x-irradiation of the adult rat. Int. J. Radiat. Biol. 2, 157-170, 1960.

48. Keulen van CJG and de Rooij DG: Spermatogonial stem cell renewal in the mouse. II. After cell loss. Cell Tiss. Kinet. 6, 337-345, 1973.

49. Keulen van CJG and de Rooij DG: The recovery from various gradiations of cell loss in the mouse seminiferous epithelium and its implications for the spermatogonial stem cell renewal theory. Cell Tiss. Kinet. 7, 549-558, 1974.

50. Lu CC, Meistrich ML and Thames HD: Survival of mouse testicular stem cells after ɣ or neutron irradiation. Radiat. Res. 81, 402-415, 1980.

51. Madhwa Raj HG and Dym M: The effects of selective withdrawal of FSH or LH on spermatogenesis in the immature rat. Biol. Reprod. 14, 489-494, 1976.

52. Main SJ, Davies RV and Setchell BP: Feedback control by the testis of gonadotrophin secretion: An examination of the inhibin hypothesis. J. Endocr. 79, 255-270, 1978.

53. Mandl AM: The radiosensitivity of germ cells. Biol. Rev. 39, 288-371, 1964.

54. McCullagh DR: Dual endocrine activity of testes. Science. 76, 19-20, 1932

55. Means AR, Fakundig JL, Huckins C, Tindall DJ and Vitale R: Follicle stimulating hormone, the Sertoli cell and spermatogenesis. Rec. Prog. Horm. Res. 32, 477-525, 1976.

56. Meistrich ML, Finch MV, Hunter N and Milas L: Protection of spermatogonial survival and testicular function by WR-2721 against high and low doses of radiation. Int. J. Rad. Oncol. Biol. Phys. 10, 2099-2107, 1984.

57. Milas L, Hunter N, Reid BO and Thames HD: Protective effect of S-2-(3amino-propylamino)ethylthiophosphoric acid against radiation damage of normal tissue and a fibrosarcoma in mice. Cancer Res. 42, 1888-1897, 1982.

58. Monesi V: Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. J. Cell Biol. 14, 1-18, 1962.

59. Monesi V: Relation between X-ray sensitivity and stages of the cell cycle in spermatogonia of the mouse. Radiat. Res. 17, 809-838, 1962.

60. Mulder E, Peters MJ, van Beurden WMO, Galdieri M, Rommerts FFG, Janszen FHA and van der Molen HJ: Androgen receptors in isolated cell preparations obtained from rat testicular tissue. J. Endocr. 70, 331-332, 1976.

61. Nelson WO and Merckel CE: Maintenance of spermatogenesis in hypophysectomized mice with androgenic substances. Proc. Soc. Exptl. Biol. Med. 38, 737-740, 1938.

62. Oakberg EF: Sensitivity and time of degeneration of spermatogenic cells irradiated in various stages of maturation in the mouse. Radiat. Res. 2, 369-391, 1955.

63. Oakberg EF: Degeneration of spermatogonia of the mouse following exposure to X-rays and stages in the mitotic cycle at which cell death occurs. J. Morph. 97, 39-54, 1955.

64. Oakberg EF: γ ray sensitivity of spermatogonia of the mouse. J. Exp. Zool. 134, 343-356, 1957.

65. Oakberg EF: Initial depletion and subsequent recovery of spermatogonia of the mouse after 20 R of γ rays and 100, 300, and 600 R of X-rays. Radiat. Res. 11, 700-719, 1959.

66. Oakberg EF: The effects of dose rate and quality of radiation on the dynamics and survival of the spermatogonial population of the mouse. Jap. J. Genet. (Suppl.) 40, 119-127, 1964.

67. Oakberg EF: Spermatogonial stem cell renewal in the mouse. Anat. Rec. 169, 515-532, 1971.

68. Oakberg EF: Differential spermatogonial stem cell survival and mutation frequency. Mut. Res. 50, 327-340, 1978.

69. Oakberg EF and Huckins C: Spermatogonial stem cell renewal in the mouse as revealed by H^3-thymidine labeling and irradiation. J. Endocr. 60, 287-293, 1974.

70. Orth J and Christensen AK: Autoradiographic localization of specifically bound I^{125}-labelled follicle stimulating hormone on spermatogonia of the rat testis. Endocrinology. 103, 1944-1951, 1978.

71. Partington M, Craig AW and Jackson H: The effect of radiation on spermatogenic cells in the rat and mouse. Brit. J. Radiol. 35, 713-718, 1962.

72. Patt HM, Tyree EP, Straube RL and Smith DE: Cysteine protection against X-irradiation. Science. 110, 213-214, 1949.

73. Phillips TL: Sensitizers and protectors in clinical oncology. Sem. Oncol. 8, 65-82, 1981.

74. Purvis K, Calandra R, Sander S and Hansson V: Androgen binding proteins and androgen levels in the human testis and epididymis. Int. J. Androl. 1, 531-548, 1978.

75. Régaud C and Blanc J: Action des rayons X sur les diverses generations de la lignee spermatique. Extrème sensibilité des spermatogonies a ces rayons. C. R. Seances Soc. Biol. 61, 163-165, 1906.

76. Rooij DG de: Spermatogonial stem cell renewal in the mouse. I. Normal situation. Cell Tiss. Kinet. 6, 281-287, 1973.

77. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Response of stem cells in the mouse testes to fission neutrons of 1 MeV mean energy of 300 KV X-rays. Methodology, dose-response studies, relative biological effectiveness. Radiat. Res. 67, 56-68, 1976.

78. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean energy or 300 KV X-rays. Radiat. Res. 71, 579-592, 1977.

79. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean energy. Effect of the fractionation interval. Radiat. Res. 79, 289-297, 1979.

80. Sanborn BM, Elkington JSH, Chowdhury M, Tcholakian RK and Steinberger E: Hormonal influences on the level of testicular androgen binding activity: Effect of FSH following hypophysectomy. Endocrinology. 96, 304-312, 1975.

81. Schinz HR and Slotopolsky B: Der Röntgen Hoden. Ergebn. Med. Strahlenforsch. 1, 443-526, 1925.

82. Sharpe RM: Intratesticular factors controlling testicular function. Biol. Reprod. 30, 29-49, 1984.

83. Shaver SL: X-irradiation injury and repair in the germinal epithelium of male rats. I. Injury and repair in adult rats.; II. Injury and repair in immature rats. Am. J. Anat. 92, 391-450, 1953.

84. Sheridan W: The effects of the time interval in fractionated X-ray treatment of mouse spermatogonia. Mut. Res. 13, 163-169, 1971.

85. Smith PE: Hypophysectomie and a replacement therapy in the rat. Am. J. Anat. 45, 205-256, 1930.

86. Smith JA and Martin L: Do cells cycle ? Proc. Natl. Acad. Sci. USA 70, 1263-1267, 1973.

87. Steinberger E: Hormonal control of mammalian spermatogenesis. Phys. Rev. 51, 1-22, 1971.

88. Steinberger E and Duckett GE: Hormonal control of spermatogenesis. J. Reprod. Fertil. (Suppl.) 2, 75-87, 1967.

89. Steinberger A, Heindel JJ, Lindsey JN, Elkington JSH, Sanborn BM and Steinberger E: Isolation and culture of FSH responsive Sertoli cells. Endocr. Res. Commun. 2, 261-272, 1975.

90. Steinberger A and Steinberger E: Secretion of an FSH inhibiting factor by cultured Sertoli cells. Endocrinology. 99, 918-921, 1976.

91. Steinberger A and Steinberger E: Inhibition of FSH by a Sertoli cell factor in vitro. In: The testis in normal and infertile men (ed. by Troen P and Nankin HR, Raven Press New York), 271-279, 1977.

92. Thumann A and Bustos-Obregon E: An "in vitro" system for the study of rat spermatogonial proliferative control. Andrologia. 10, 22-25, 1978.

93. Tindall DJ and Means AR: Concerning the hormonal regulation of androgen binding protein in rat testis. Endocrinology. 99, 809-818, 1976.

94. Tindall DJ, Miller DA and Means AR: Characterization of androgen receptor in Sertoli cell-enriched testis. Endocrinology. 101, 13-23, 1977.

95. Walsh EL, Cuyler WK and McCullagh DR: Physiologic maintainance of male sex glands; Effect of androtin on hypophysectomized rats. Am. J. Phys. 107, 508-512, 1934.

96. Weddington SC, Hansson V, Ritzen EM, Hagenas L, French FS and Nayfeh SN: Sertoli cell secretory function after hypophysectomy. Nature 254, 145-146, 1975.

97. Withers HR, Hunter N, Barkley HT and Reid BO: Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testes. Radiat. Res. 57, 88-103, 1974.

98. Yahas JM and Storer JB: Chemoprotection against three modes of radiation death in the mouse. Int. J. Radiat. Biol. 15, 233, 1969.

CHAPTER 3

OUTLINE OF THE STUDY

Although the spermatogonial stem cells appear to be very radioresistant cells (9,11,26,27,28,29,34,40), their state of radioresistance is not a permanent one (11,34,36). The implication of variations in radiosensitivity of stem cells, suggests particular vulnerability at particular intervals. A survey of the literature (Fig. 3.1) of the survival of testicular stem cells after fractionated irradiation, however, shows conflicting results. Withers et al. (40) and Lu et al. (21) showed enhanced stem cell survival when the dose was split into two equal fractions, whereas

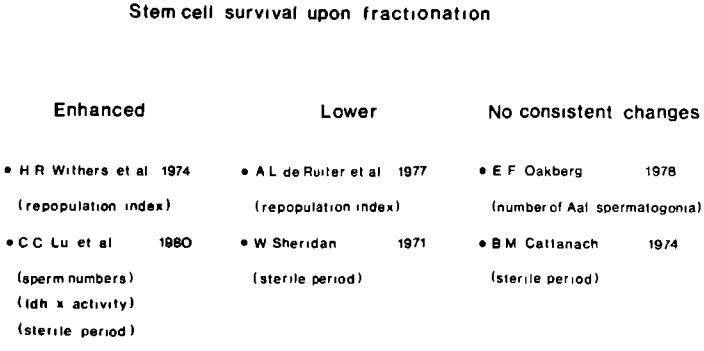


FIG. (3.1) *Stem cell survival after split dose irradiation as found by different authors.*

Oakberg (27) and Cattanach (4) reported no consistent changes.

It has been shown (34) that in the mouse a first dose of irradiation triggers the stem cells to pass into a very radiosensitive phase of their cell cycle after 16 hours. A second dose of irradiation, however, will destroy these sensitive stem cells (11), suggesting that depletion of stem cells will occur (34,36). The factor responsible for triggering these cells in the radiosensitive phase is still unknown. It is possible that the

change in radiosensitivity can be explained by the change in the phase of the cell cycle of the stem cells (14,26,34,40). Each phase of the cell cycle has a different sensitivity to irradiation (25). This phenomenon was also described by Sinclair and Morton (38). They found that, with the exception of mitosis, cells are most radiosensitive just towards the end of the G_1-phase. They then become progessively more resistant as they progress through the S-phase, but become more sensitive when they pass into the G_2-phase before the next mitosis. The most radiosensitive point in the cycle is when cells are completing G_1 and commencing S-phase.

From a theoretical point of view, hormones could be considered to be able to reduce or to avoid radiation damage, arresting cell division in the radioresistant phase. This could protect stem cells from depletion, resulting in preservation of reproductive function. By inhibiting cell division, there may also be a greater opportunity for repair of radiation induced damage. While proof of this hypothesis is lacking, its validity can be tested by interrupting the pituitary-gonadal axis by hormones during testicular irradiation.

To interrupt the pituitary-gonadal axis during or prior to irradiation procedures, two drugs with known hormonal inhibitory effects on spermatogenesis were tested. The first drug Danazol® (a synthetic steroid derived from ethisterone) has been reported (2,7,8,10,30,31,32,37) to possess inhibiting effects on pituitary gonadotropins. The second drug, Zoladex® (ICI 118,630), is a LHRH analogue. These analogues, are thought to have direct effects on the LHRH receptors in the pituitary gland, resulting in low serum concentrations of gonadotropins probably by down regulation (1,6,13,18,19,35) and desensitization.

Since much is known about morphological aspects of spermatogenesis and the endocrine regulation of spermatogenesis in the rat (39), it was decided to use this species in the experiments performed. Because the "As" model of spermatogenesis of Huckins fits best with the present knowledge of spermatogonial proliferation and stem cell renewal in the rat (chapter 2.2.1), this model was applied throughout the thesis. Stem cell survival was measured by using the repopulation index (21,33,34,40) as an assay for stem cell survival.

A good correlation between this index and stem cell survival has been shown (33). Flow cytometric analyses were performed to measure the change in proliferation kinetics in the process of spermatogenesis. Flow cytometry has been developed over the last twenty years from an effort initially intended to count (3,5,12,15,24) and later to size particles (16,17,22) into a sophisticated analytical tool, which allows rapid quantitative evaluation of multiple properties of the individual cell. The haploid cells as measured by the flow cytometer correspond to spermatids. Sperm head counting by microscope was also used as an assay in evaluating stem cell survival (21,23). Meistrich et al. (23) showed the relationship between the rate of recovery of sperm production and stem cell survival. The level of sperm production after irradiation is also directly related to fertility. Irradiation was given in a single dose or split into fractions with or without hormonal treatment. To evaluate the effect on the different cell types, histological counting of the number of spermatogonia (type A, intermediate and type B) and the number of preleptotene spermatocytes was performed using the classification method as proposed by Leblond and Clermont (20). Serum gonadotropins and testosterone concentrations were measured to evaluate the hormonal suppression.

References

1. Ahmed SR, Grant J, Shalet SM, Howell A, Chowdhury SD, Weatherson T and Blacklock NJ: Preliminary report on use of depot formulation of LHRH analogue ICI 118,630 (Zoladex) in patients with prostatic cancer. Br. Med. J. 290, 185-187, 1985.

2. Barbieri RL, Canick JA, Makris A, Todd RB, Davies IJ and Ryan KJ: Danazol inhibits steroidogenesis. Fert. Steril. 28, 809-813, 1977.

3. Brecher G, Schneiderman MA and William GE: Evaluation of an electronic red cell counter. Am. J. Clin. Pathol. 26, 1439-1449, 1956.

4. Cattanach BM: Spermatogonial stem cell killing in the mouse following single and fractionated X-ray doses, as assessed by length of sterile period. Mut. Res. 25, 53-62, 1974.

5. Crosland-Taylor PJ. A device for counting small particles suspended in a fluid through a tube. Nature. 171, 37-38, 1953.

6. Cusan L, Auclair C, Belanger A, Ferland L, Kelly PA, Seguin C and Labrie F Inhibitory effects of long term treatment with a luteinizing hormone releasing hormone agonist on the pituitary-gonadal axis in male and female rats. Endocrinology 104, 1369-1376, 1979.

7. Dixit VP, Agrawal M and Varma M· Male contraception properties of a new synthetic steroid derivative (Danazol) in Rattus rattus Rufescens. Endocrinologie. 77, 30-38, 1981.

8. Dmowski WP, Scholer HFL, Mahesh VB and Greenblatt RB· Danazol-A synthetic steroid derivative with interesting physiologic properties. Fert. Steril. 22, 9-18, 1971.

9. Dym M and Clermont Y· Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. Am. J. Anat. 128, 265-282, 1970.

10. Eldridge JC, Dmowski WP and Mahesh VB: Effects of castration of immature rats on serum FSH and LH, and of various steroid treatments after castration. Biol Reprod. 10, 438-446, 1974.

11. Erickson BH: Effect of Co^{60} γ radiation on the stem and differentiating spermatogonia of the post pubertal rat. Radiat. Res. 68, 433-448, 1976.

12. Gucker FT, O'Konski CT, Pickard HB, Pitts JN: A photoelectic counter for colloidal particles. J. Am. Chem. Soc. 69, 2442, 1947.

13. Heber D and Swerdloff RS: Gonadotropin-releasing hormone analog and testosterone synergistically inhibit spermatogenesis. Endocrinology. 108, 2019-2021, 1981.

14. Huckins C: Behavior of stem cell spermatogonia in the adult rat irradiated testis. Biol. Reprod. 19, 747-760, 1978.

15. Kamentsky LA, Melamed MR, Derman H: Spectrophotometer: New instrument for ultra rapid cell analysis. Science 150, 630-631, 1965.

16. Kubitschek HE: Electronic counting and sizing of bacteria. Nature 182, 234-235, 1958.

17. Kubitschek HE: Electronic measurement of particle size. Research (London) 13, 128-135, 1960.

18. Labrie F, Auclair C, Cusan L, Kelly PA, Pelletier G and Ferland L: Inhibitory effects of LHRH and its agonists on testicular gonadotropin receptors and spermatogenesis in the rat: Endocrine approach to male contraception. Int. J. Androl. (Suppl.) 2, 303-318, 1978.

19. Labrie F, Cusan L, Séguin C, Bélanger A, Pelletier G, Reeves J, Kelly PA, Lemary A and Raynaud JP: Antifertility effects of LH-RH agonists in the male rat and inhibition of testicular steroidogenesis in man. Int. J. Fertil. 25, 157-170, 1980.

20. Leblond CP and Clermont Y: Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55, 548-573, 1952.

21. Lu CC, Meistrich ML and Thames HD: Survival of mouse testicular stem cells after γ or neutron irradiation. Radiat. Res. 81, 402-415, 1980.

22. Mattern CFT, Brackett FS and Olson BJ: The determination of number and size of particles by electical gating: Blood cells. J. Appl. Physiol. 10, 56-70, 1957.

23. Meistrich ML, Hunter NR, Suzuki N, Trostle PK and Withers HR: Gradual regeneration of mouse testicular stem cells after exposure to ionizing radiation. Radiat. Res. 74, 349-362, 1978.

24. Moldavan A: Photo-electric technique for the counting of microscopical cells. Science. 80, 188-189, 1934.

25. Monesi V: Relation between X-ray sensitivity and stages of the cell cycle in spermatogonia of the mouse. Radiat. Res. 17, 809-838, 1962.

26. Oakberg EF: Effects of radiation on the testis. In Handbook of Physiology Section 7. Endocrinology Vol. V (ed. Greep RO et al.) Am. Phys. Soc. Washington, 233-243, 1975.

27. Oakberg EF: Differential spermatogonial stem cell survival and mutation frequency. Mut. Res. 50, 327-340, 1978.

28. Oakberg EF and Huckins C: Spermatogonial stem cell renewal in the mouse as revealed by H^3-thymidine labeling and irradiation. J. Endocr. 60, 287-293, 1974.

29. Partington M, Craig AW and Jackson H: The effect of radiation on spermatogenic cells in the rat and mouse. Br. J. Radiol. 35, 713-718, 1962.

30. Paulsen CA and Leonard JM: Clinical trials in reversible male contraception I Combination of Danazol plus Testosterone. In Regulatory mechanisms of male reproductive Physiology (ed. Spilman CH, Lobl TJ, Kirton KT, Excerpta Medica-Amsterdam, The Netherlands). 197-211, 1976.

31. Pedroza E, Vilchez-Martinez JA, Arimura A and Schally AV: Danazol effects on gonadotropin basal levels and pituitary responsiveness to LH-RH in immature male rats. Contraception 17, 61-69, 1978.

32. Potts GO, Beyler AL and Schane HP: Pituitary gonadotropin inhibitory activity of Danazol. Fert. Steril. 25, 367-372, 1973.

33. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Response of stem cells in the mouse testes to fission neutrons of 1 MeV mean energy of 300 KV X-rays. Methodology, dose-response studies, relative biological effectiveness. Radiat. Res. 67, 56-68, 1976.

34. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean energy or 300 KV X-rays. Radiat. Res. 71, 579-592, 1977.

35. Schally AV, Kastin AJ, Coy DH: Luteinizing hormone-releasing hormone and its analogues: Recent basic and clinical investigations. Int. J. Fertil. 21, 1-30, 1976.

36. Sheridan W: The effects of the time interval in fractionated X-ray treatment of mouse spermatogonia. Mut. Res. 13, 163-169, 1971.

37. Sherins RJ, Gandy HM, Thorslund TIV and Paulsen CA: Pituitary and testicular function studies. I Experience with a new gonadal inhibitor, 17α-Pregn-4-en-20-yno-(2,3-d) isoxazol-17-ol (Danazol). J. Clin. Endocr. Metab. 32, 522-531, 1971.

38. Sinclair WK and Morton RA: X-ray sensitivity during the cell generation cycle at cultured chinese hamster cells. Radiat. Res. 29, 450-474, 1966.

39. Steinberger E: Hormonal control of mammalian spermatogenesis. Phys. Rev. 51, 1-22, 1971.

40. Withers HR, Hunter N, Barkley HT and Reid BO: Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testes. Radiat. Res. 57, 88-103, 1974.

CHAPTER 4

A COMPUTER MODEL OF SPERMATOGENESIS IN THE RAT CORRELATION WITH FLOW CYTOMETRIC DATA BASED ON AUTORADIOGRAPHIC CELL-CYCLE PROPERTIES

M.J.P.G. van Kroonenburgh, H.J. van Gasteren,
J.L. Beck and C.J. Herman

(Published in Cell and Tissue Kinetics 19, 171-177, 1985)

ABSTRACT. A computer model of rat spermatogenesis was created, based on autoradiographic studies of durations of the phases of the cell cycle (G_1, S, G_2 and mitotic phases) of each germ-cell type. With this model it is possible to predict and to gain insight into the distinct changes of the DNA content occurring during the normal process of spermatogenesis. The relative proportions of haploid, diploid, S phase and tetraploid germ cells with increasing age of the rats were calculated. Calculated and actual experimental flow cytometry data were compared to test the accuracy of the model, and these show good agreement. The present work demonstrates that single-parameter DNA analysis of testicular cells is primarily a reflection of germ cells in the spermatocyte and spermatid stages of development, and of non-germ cells. The FCM single-parameter DNA analysis of testicular cells is relatively insensitive to changes in the stem cell and spermatogonial stages of germ-cell development.

INTRODUCTION

The currently most acceptable model of spermatogonial stem cell renewal and differentiation to spermatids in rat testis is the model proposed by Huckins (13,15) and Huckins and Oakberg (16). In this model, the spermatogenic stem cells (As cells) renew themselves and also give rise to conjoined pairs of spermatogonia (Ap cells). Continued proliferation of the Ap spermatogonia gives rise to longer and longer chains of A-aligned (Aal) cells. These Aal cells appear to transform into A1 spermatogonia which will go on to proliferate and to differentiate into spermatocytes and spermatids. The duration of the different phases of the cell cycle of these spermatogenic cells has been well documented with the aid of autoradiography (10,11,14).

In studying spermatogenesis, physiological, pharmacological, hormonal and radiobiological effects may affect one or more of the stages of germ-cell development from stem cell to spermatid. These effects are reflected in changes of the DNA distribution of testicular cells as measured by flow cytometry (FCM; 1,2,3,7,18). With this technique, it is possible to quantify the percentage of cells in the different phases of the spermatogenic cell cycle by using fluorescent dyes, which bind to cellular DNA (8,9,22). However, the stage(s) of germ cell responsible for the observed DNA changes can not be directly identified using FCM alone. Therefore a computer model of spermatogenesis, based on autoradiographic data in the literature and reflecting the phases of the cell cycle, has been developed to refine the recognition of germ-cell populations defined by FCM DNA measurements.

MATERIALS AND METHODS

Input data

The populations of spermatogenic cells were analysed separately by compartments, each compartment consisting of cells in the same

phase of the cell cycle. These compartments and the time that each cell type spends in the compartment are well-documented in the literature (Fig. 4.1, based on: data from Hilscher et al. (10,11) and Huckins (14)). Those data were found using autoradiographic techniques and were originally expressed as the length (in hr) of the different phases of the cell cycle (G_1, S, G_2 and M). In this study, these times have been considered to be independent of age and physical condition of the rat. It has been recognized histologically that spermatogenesis in normal rats includes several steps in which germ cells spontaneously degenerate, such that the seminiferous epithelium yields fewer spermatozoa than theoretically possible. There are three critical points in differentiation where extensive cell loss occurs (15). The first is during spermatogonial

		G_1	S	G_2 + M	
A	stemcell	⩾18 0	24 5	17 5	
	↓				
A	paired (1)	13 0	25 0	16 5	
A	aligned (2)	13 0	25 0	16 5	
A	aligned (4)	13 0	25·0	16 5	
A	aligned (8)	13 0	25 0	16 5	
A	aligned (16)	13 0	25 0	16 5	
A1	spermatogonia	108 5	19 5	13 0	
A2	spermatogonia	7 5	20 5	11 0 (65%)	
A3	spermatogonia	11 0	21 5	10 0 (38%)	
A4	spermatogonia	10 0	23 0	9 0 (9%)	
	↓				
Intermediate		10 0	24 0	8 0	
	↓				
B	spermatogonia	9 0	25 5	7 5	
	↓				
	spermatocyt 1	88 8	26 0	372 0 (12%)	
	spermatocyt 2			6 0 (15%)	
	↓				
	spermatids				532 8 (18%)

FIG. (4.1) *Duration (hr) of G_1, S, G_2 and M phases of cell cycle in the maturation stages of spermatogenesis as found with autoradiographic techniques (10,11,14). Figures in parentheses indicate the percentage of degeneration in that stage of development, a normal process during spermatogenesis.*

development, involving primarily type A2 and A3 spermatogonia.

It has been shown that type A-spermatogonia degenerate during their mitotic peaks (4,15). The second critical step is during meiotic division of spermatocytes (15) and finally spermatids degenerate during the acrosome phase of spermiogenesis (20,21). The percentages of degeneration are given in Fig. 4.1 Besides the spermatogenic cells, non-germ cells such as Sertoli cells, Leydig cells, endothelial cells, etc. are present in the testis. The percentage of non-germ cells was variable in our model but generally adjusted to a stable value of 10% (6).

Computer modelling

Spermatogenesis can be represented as a family tree originating from a single stem cell born at t=0. Each branch of the tree represents a cell and is divided lengthwise into four segments which correspond to the four phases of the cell cycle. Every t hr a fixed number of stem cells begins spermatogenesis and develops according to Fig. 4.1 as a growing number of cells as time continues. The numbers of cells in each compartment can be computed and summed. The time axis is divided into discrete intervals (Δt). In the present study, Δt was chosen as 5 hr. Any arbitrary Δt can be chosen for modelling purposes, if it is short in relation to the times given in Fig. 4.1. The number of germ cells in the various stages of development and in the different phases of the cell cycle as a function of time, is obtained by summing the contribution of each individual stem cell, which basically gives an error function-shaped curve. Cells which degenerate (according to the percentages in Fig. 4.1) are eliminated in the model. The development of the germ cell population has been modelled using the above algorithm as a function of age of the rat. The model is written in FORTRAN for a PDP 11/34 computer operating under RT 11 (Digital Equipment Corp., Maynard, Mass., U.S.A.).

Output data

The amount of DNA per cell can be expressed as the c value, representing the amount of DNA in a gamete. The G_1-phase cells and G_2+M-phase cells contain a 2c and 4c amount of DNA, respectively. Because cells in the G_2 and M phases of the cell cycle have the same DNA content, they cannot be distinguished by single-parameter FCM analysis. Thus, for analysis in the present study, they are combined into a single phase (G_2+M) with a 4c DNA content. Haploid cells (spermatids) have a 1c DNA content. By summing the total numbers of cells in each germ-cell development stage in each phase of the cell cycle, the relative proportions of the total germ-cell population in each phase of the DNA histogram can be computed. These data can be directly compared with real DNA data to verify the model.

RESULTS

The calculated DNA content frequency distributions in percentages of haploid (1c), diploid (2c), S-phase and tetraploid (4c) cells as a function of the age of the rat are presented in Fig. 4.2 The time axis of this figure is calibrated according to the results of Clausen et al. (2; see also Fig. 4.3). Examination of these figures shows that the model predicts a number of important features of germ-cell development. For example, after day 22, a rapid increase in haploid cells occurs, reaching a level of 70.1% of total testicular cells beyond day 52 (Fig. 4.2). The increase in tetraploid cells precedes the completion of meiosis, which in the model occurs on day 20 (Fig. 4.2). The tetraploid cells reach a maximum percentage of 52.0%. Alterations in the proportion of diploid cells and cells in the S-phase from rats of different ages are presented in Fig. 4.2.

At the time of birth, a peak of cells in the S-phase is observed. The relative contribution of cells in the various stages of maturation to a total DNA histogram is summarized in Fig. 4.4 The calculated percentages of cells with 1c, 2c, S and 4c DNA content correlate well with observed FCM data from mature rats which have not been treated with hormones, drugs, etc. (data not shown). For comparison, data from FCM analysis of rat testis at different ages

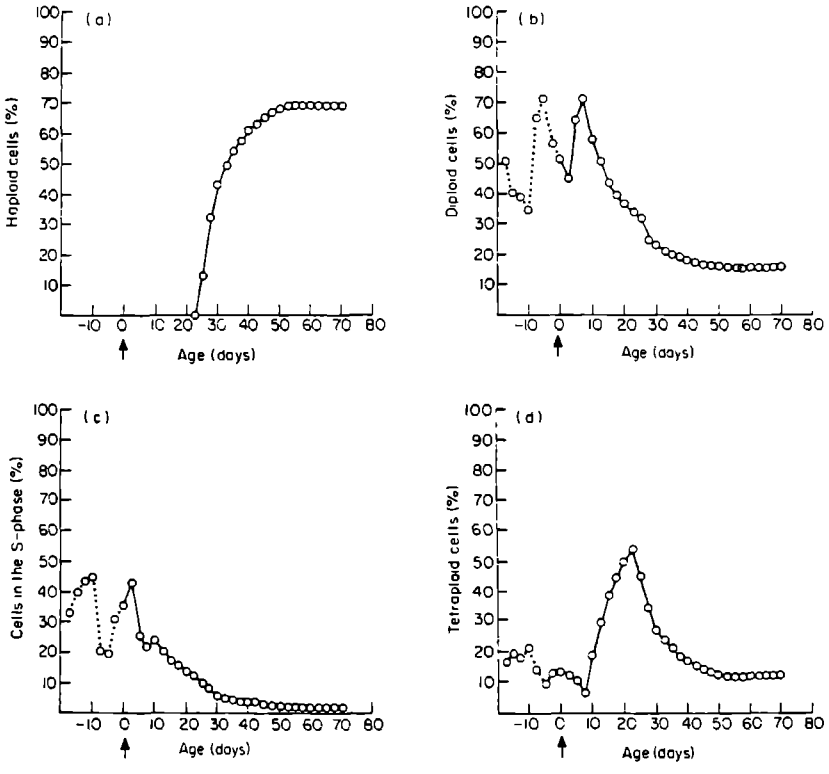


FIG. (4.2) *Percentages of haploid (a), diploid (b), S-phase (c) and tetraploid (d) cells in the germ-cell population of rat testis as a function of animal age. Birth=0 (↑). Data based on the computer simulation described in the text.*

are presented in Fig. 4.3 (data from Clausen et al., 2). Comparison of Figs. 4.2 and Fig. 4.3 shows that the model predicts relatively accurately the distribution of cells found experimentally.

DISCUSSION

The computer model of rat spermatogenesis presented in the present paper involves several simplifications. Firstly, the

relative amount of non-germ cells is adjusted to 10% of the total population obtained in a suspension of testicular cells. Secondly, the data on duration of the phases of the cell cycle presented in Fig. 4.1 are considered to be independent of the age of the rat. Thirdly, our model considers spermatogenesis to proceed in a population of cells whose proliferative rate is independent of any

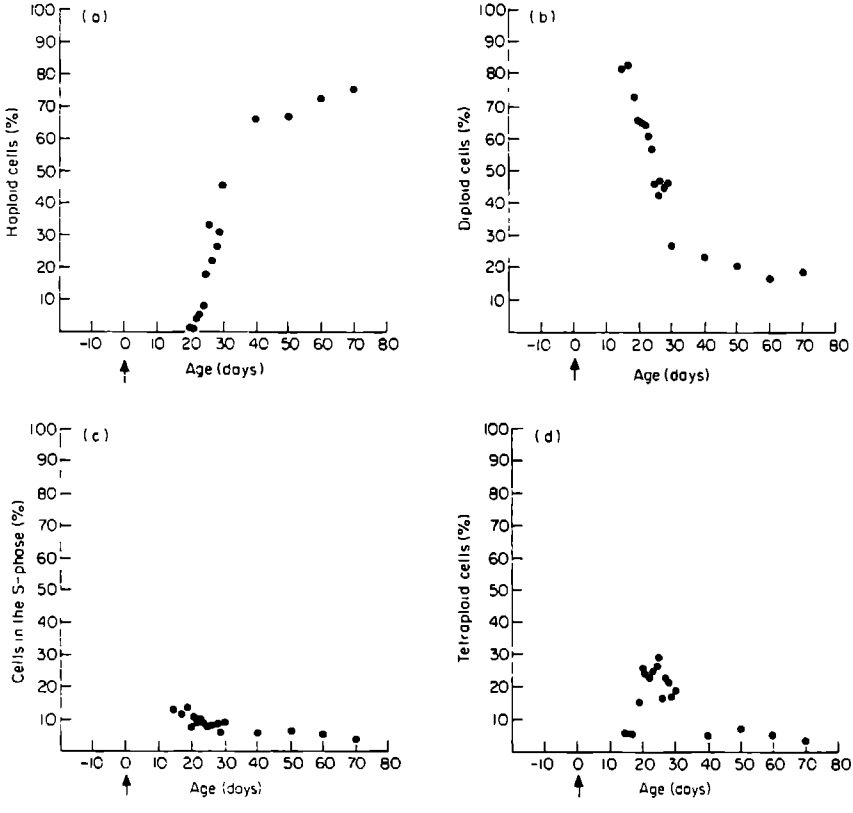


FIG. (4.3) *Percentages of haploid (a), diploid (b), S-phase (c) and tetraploid (d) cells in the germ-cell population of rat testis as function of animal age. Birth =0 (↑). Data based on FCM studies from Clausen et al.* (2).

homoeostatic mechanism.

Concerning this last point, labelling studies suggest that the duration of spermatogenesis from beginning of spermatogonial differentiation to spermatozoal release is constant for each species (5). However, there is evidence that the process proceeds more rapidly in juvenile animals (12). Despite the limitations of the model, predicted values of cell-cycle distribution in testicular cell suspensions fit very well with experimentally found values by Clausen et al. (2). Comparison of FCM data from Clausen et al. (2) with our calculated data generally shows a good overall agreement. A discrepancy between experimental and calculated data is noticed in the tetraploid curves however (compare Fig. 4.2 and 4.3). The percentage of 4c cells at their peak, approximately 25 days after birth, is significantly different. According to our model, a transit time of the primary spermatocytes of 172 hours instead of 372 hours in the G_2+M phase (Fig. 4.1) would give a much better correlation between the predicted and observed tetraploid cell curves.

With the computer model it is also possible to predict DNA distribution changes after irradiation. Irradiation can selectively kill spermatogonia (17,19) leaving the stem cell intact. So depletion of spermatogonia will result in disappearance of spermatogenic cells at a specific stage of the maturation sequence and eventually in loss of sperm at a predictable time following irradiation. The surviving stem cells must eventually repopulate the germinal epithelium in sequence, according to Fig. 4.1 So fluctuations in the DNA distributions can be expected after irradiation (7) similar to the dotted lines shown in Fig. 4.2

The most important conclusion from the present study can be drawn from an examination of Fig. 4.4. Fig. 4.4 shows that the 4c population as measured by FCM consists mainly (98%) of primary spermatocytes, due to their extended stay in G_2+M phase (372 hr. see Fig. 4.1). The S-phase cells demonstrated by FCM are nearly equally divided between spermatogonia (43%) and preleptotene spermatocytes (57%). Spermatogonia are present in the 2c and 4c region in a relatively low percentage (0.2%). It is clear that routine single-parameter DNA analysis of testis cells is primarily a reflection of germ cells in spermatocyte and spermatid stages of

		2c	S	4c	1c
A	stemcell				
	↓				
A	paired (1)				
A	aligned (2)				
A	aligned (4)				
A	aligned (8)				
A	aligned (16)				
	↓				
A1	spermatogonia	0·2%	1 3%	0 2%	
A2	spermatogonia				
A3	spermatogonia				
A4	spermatogonia				
	↓				
Intermediate					
	↓				
B	spermatogonia				
	↓				
	spermatocyt 1	5·0%	1 7%	9·8%	
	spermatocyt 2	0·8%			
	↓				
	spermatids				71 0%
	non-germ	10·0%			
	Total %	16·0%	3·0%	10·0%	71·0%

FIG. (4.4) *Theoretical DNA content (c value) distribution as percentages of the whole population of germ cells as predicted by the simulation model, in the adult (> 52 days old) rat. Total percentages would be observed as a single-parameter DNA histogram.*

development and of non-germ, primarily 2c cells. The only single-parameter DNA measurement which may be sensitive enough to reflect small changes in the stem-cell and spermatogonial compartments is the S-phase, where cells in the early stages of spermatogenesis are represented approximately equally with spermatocytes. Thus, an improved technique to examine the individual germ-cell developmental stages separately is highly desirable. This will be the subject of a subsequent report.

References

1. Clausen OPF, Purvis K and Hansson V: Application of Micro-flow fluorometry to studies of meiosis in the male rat. Biol. Reprod. 17, 555-560, 1977.

2. Clausen OPF, Purvis K and Hansson V: Assesment by micro flow fluorometry of the prurity of interstitial cell suspensions from the rat testis. J. Reprod. Fert. 52, 387-390, 1978.

3. Clausen OPF, Parvinen M and Kirkhus B: Stage related variations in DNA fluorescence distribution during rat spermatogenic cycle measured by flow cytometry. Cytometry. 6, 421-425, 1982.

4. Clermont Y: Qualitative analysis of spermatogenesis of the rat. A revised model for the renewal of spermatogonia. AM. J. Anat. 111, 111-129, 1962.

5. Clermont Y: Kinetics of spermatogenesis in mammals:Seminiferous epithelium cycle and spermatogonial renewal. Phys. Rev.52, 198-236, 1972.

6. Hacker U, Schumann J and Göhde W: Effects of acute ɣ-irradiation on spermatogenesis as revealed by flow cytometry. Acta Radiol. Oncol. 19, 361-368, 1980.

7. Hacker U, Dissertation: Biologische dosimetrie ionisierender strahlung am modell der spermatogenese der maus. Münster, W. Germany. 1981.

8. Hamilton VT, Habersett MC and Herman CJ: Flow microfluorometric analysis of cellular DNA: Critical comparison of mithramycin and propidium iodide. J.Hist. Cytochem. 28, 1125-1128, 1980.

9. Herman CJ, Vooys GP, Baak JPA and Boon ME: Quantitative cytologic and histologic techniques to assist in cancer evaluation. In Jasmin,G., Proschekl. (ed.). Methods Achiev. Exp. Pathol. 11, 73-95, 1984.

10. Hilscher B, Hilscher W and Maurer W: Autoradiographische Untersuchungen ueber den Modus der Proliferation und Regeneration des Samenepithels der Wistar Ratte. Z. Zellforsch. 94, 593-604, 1969.

11. Hilscher W und Hilscher B: Autoradiographische Untersuchungen ueber die Kinenetik der Spermatogenese der Wistar ratte unter Berücksichtigung der Spermacytogenese. Z. Zellforsch. 96, 625-650, 1969.

12. Huckins C: The initiation of spermatogenesis in the testis of the Wistar Albino rat (Ph.D.Thesis) Montreal: Mc Gill University 1965.

13. Huckins C: The spermatogonial stem cell population in adult rats. I Their morphology, proliferation and maturation. Anat. Rec. 169, 533-558, 1971.

14. Huckins C: The spermatogonial stem cell population in adult rats. II a radioautografic analysis of their cell cycle properties. Cell Tiss. Kinet. 4, 313-334, 1971.

15. Huckins C: The morphology and Kinetics of spermatogonial degeneration in normal adult rats. Analysis in using a simplified classification of the germinal epithelium. Anat. Rec. 190, 905-926, 1978.

16. Huckins C and Oakberg EF: Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules I The normal testes. Anat. Rec. 192, 519-524, 1978.

17. Jones EA: Number of spermatogonia after X-irradiation of the adult rat. Int. J. Rad. Biol. 2, 157-170, 1960.

18. Libbus BL and Schuetz AW: Analysis of the progression of meiosis in dispersed rat testicular cells by flow cytofluorometry. Cell Tiss. Kinet. 11, 377-391, 1978.

19. Partington M, Craig AW and Jackson H: The effect of radiation on spermatogenic cells in the rat and the mouse. Br. J. Radiol. 35, 713-718, 1962.

20. Roosen-Runge E: Untersuchungen ueber die Degeneration Samenbildender zellen in der normaler Spermatogenese der Ratte. Z. Zellforsch. Mikros. K. Anat. 41, 221-235, 1955.

21. Russell LD and Clermont Y: Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. Anat. Rec. 187, 347-366, 1976.

22. Tannenbaum E, Cassidy M, Alabaster O and Herman C: Measurement of cellular DNA mass by flow microfluorometry using a biological internal standard. J. Histochem. Cytochem. 26, 145-148, 1978.

CHAPTER 5

DNA ANALYSIS AND SORTING OF RAT TESTIS CELLS USING TWO-PARAMETER FLOW CYTOMETRY

M.J.P.G. v. Kroonenburgh, J.L. Beck, J.W. Scholtz,
U. Hacker-Klom and C.J. Herman

(Published in Cytometry 6, 321-326, 1985)

ABSTRACT. By use of two-parameter flow cytometry of rat testis cell suspensions stained with mithramycin for DNA (the peak amplitude of the fluorescence signal versus total fluorescence intensity integrated over time), eight cell compartments could be distinguished without pre-enrichment of the samples. Cells in these compartments were identified by sorting and subsequent microscopic examination.

INTRODUCTION

Flow cytometric (FCM) analysis of human (13,17) and animal (2,3,4,7,8,11,15) testicular cells has primarily been done using the single parameter of total DNA stainability. Using this analysis, five distinct populations of cells can be identified. Two populations with a DNA index (DI) of approximately 0.5 (1c) represent round and elongated spermatids. At a DNA index of 1.0 (2c), cells with a diploid DNA content are seen. These cells include spermatogonia, preleptotene spermatocytes and non-germ cells such as Sertoli and Leydig cells, fibroblasts, endothelial cells, and macrophages. Cells with S and G_2+M levels of DNA are primary spermatocytes and spermatogonia (12). Meistrich and associates (12) reported distinguishing seven subpopulations of testicular cells that were identified by FCM and cell sorting. Because of the highly complex effects of both physiological and pharmacological stimuli on the subpopulations of germ cells, a more detailed analysis of these subpopulations would be useful in FCM measurement of changes in testicular function. A possible solution to this problem is offered by the fact that the chromatin structure of the various germ cells as well as the size and shape of their nuclei vary widely in the different stages of maturation from spermatogonia to elongated spermatids (1). These differences can be measured using simple DNA staining if the peak and the area of the DNA fluorescence signal are analyzed as separate parameters in a two-parameter FCM system.

The aim of the present study is to identify the different populations observed by two-parameter FCM analysis of testis and thus evaluate whether this type of analysis would be more useful in giving detailed information on the various phases of the complex process of spermatogenesis in comparison with the single parameter usually used.

MATERIALS AND METHODS

All experiments were performed with 3-mo-old male outbred Wistar rats (Cpb:WU) each weighing approximately 300 gm.

Cell dispersal

The testis was dissected free from fat and connective tissue. After decapsulation, the tissue was finely minced with scissors in 15 ml phosphate buffer (pH=7.4;PO_4^{3-} 23.8 mg/ml) and sieved through a 50-µm mesh filter. The mincing and preparations were done at room temperature. This cell suspension was passed through a 25-gauge, 1-inch needle, centrifuged (370xg) for 10 min, and resuspended in 10 ml phosphate buffer by vortexing. Debris was removed by filtration through a nylon filter (pore size: 50 µm). The suspension was again centrifuged (370xg) for 10 min and the supernatant discarded. All aliquots were fixed in 50% ethanol (-20°C) and stored at 4°C until analysis.

Flow cytometry

Staining was carried out in the dark at room temperature for 30 min, as previously described (10,14). Briefly, 1 ml of cell suspension (about $3x10^6$ cells) was centrifuged 10 min (360xg) and the ethanol discarded. The cell pellet was resuspended in 1 ml staining solution consisting of 0.1 mg Mithramycin (MM: Serva Fein Biochemica, Heidelberg, W. Germany) per milliliter of 15mM $MGCl_2$ in 0.85% NaCl. Flow cytometric analysis was performed on an Ortho System 50-H (Ortho Instruments; Westwood, MA) using the 457-nm line of the argon ion laser (Spectra Physics, Mountain View, CA) at 200 mW power. A two parameter analysis was performed in a 64x64 data field, using the peak height of the fluorescence signal and the total intensity of the signal integrated over time. In some experiments, especially when studying the G_2+M (DNA index=2.0) region, the right angle light scatter was also used as a parameter.

All data collected were stored in list mode. A right angular electronic window was placed around defined regions of interest and cells were sorted onto standard 3x1-inch microscope slides, stained with haematoxylin and examined microscopically for identification of subpopulations in the sorted FCM regions (see below). Sorts and microscopic examination were done on both peak-area and scatter-area analysis to show the relationship between the two types of analysis (Fig. 5.2).

RESULTS

FCM analysis of DNA stainability of rat testicular cells is shown in Fig. 5.1. This is a one-parameter distribution based on total fluorescence per cell integrated over time (area of the signal). If the peak height of the DNA fluorescence signal of each cell is used as a second parameter, the distribution shown in Fig. 5.2 is obtained. Using this two-parameter analysis, eight cell compartments could be distinguished, as shown schematically in Fig. 5.2 For detailed study of diploid (DI=1.0) and especially of tetraploid (DI=2.0) cells, the right angle scatter was used in place of the DNA peak height in some experiments. These distributions are shown in Fig. 5.2.

For visualisation of the 8c (DI=4.0) compartment a 128x128 data field was used in place of the 64x64 field (Fig. 5.3).

Identification of the cell populations contained in the various peaks was done by sorting and subsequent microscopic examination. A sorting window set on compartments one and two reveals round and elongated spermatids, respectively. Sorting of cells belonging to compartment 3 shows a mixture of Leydig cells, Sertoli cells and fibroblasts and indicates that this compartment consists mainly of non-cycling cells. The majority of cells seen in compartment 4 are binucleated cells and were identified as secondary spermatocytes. Other cells in this compartment were preleptotene spermatocytes and some spermatogonia. Compartment 5 contains spermatogonia and clusters of spermatids. The two different compartments in the 4c region (DNA index=2.0) were identified as follows: Compartment 6

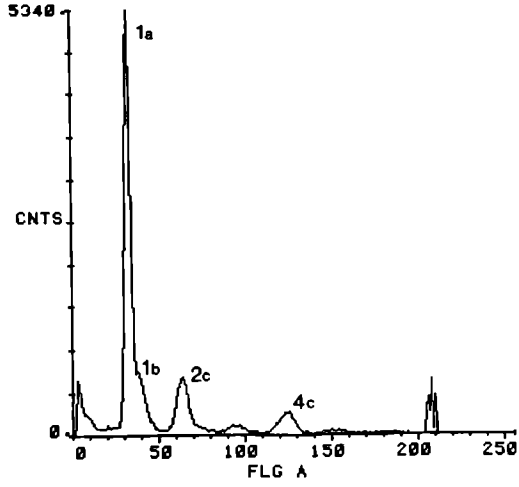


FIG. (5.1) *DNA fluorescence distribution from a single cell suspension of a rat testis. Abscissa: Total fluorescence intensity; ordinate :number of cells. The first two peaks (1a,1b) represent haploid (DI=0.5) cells, the third and fourth peaks represent diploid (DI=1.0) and 4c (DI=2.0) cells, respectively. Mithramycin stain, CV (first peak)=3.5%. Number of measured cells: 50,000.*

consists of "young" primary spermatocytes (leptotene and zygotene), cells with a relatively small nucleus, whereas in compartment 7 homogeneously pachytene primary spermatocytes are observed. Cells in compartment 8, the 8c region (DNA index=4.0), are binucleated cells with typical bean-shaped nuclei and a very dense nuclear chromatin structure (Fig. 5.5). These cells were always present. Sorting and microscopic examination on the two subpopulations found with scatter-area analysis (Fig. 5.2) in the 2c region (DNA index=1.0) reveals that cells in these subpopulations were identified as belonging to compartment 4 and 5 and compartment 3, respectively (schematically shown in Fig. 5.2). The results of FCM analysis and quantification of the eight populations of testicular cells as well as their microscopic identification are summarized in Fig. 5.4.

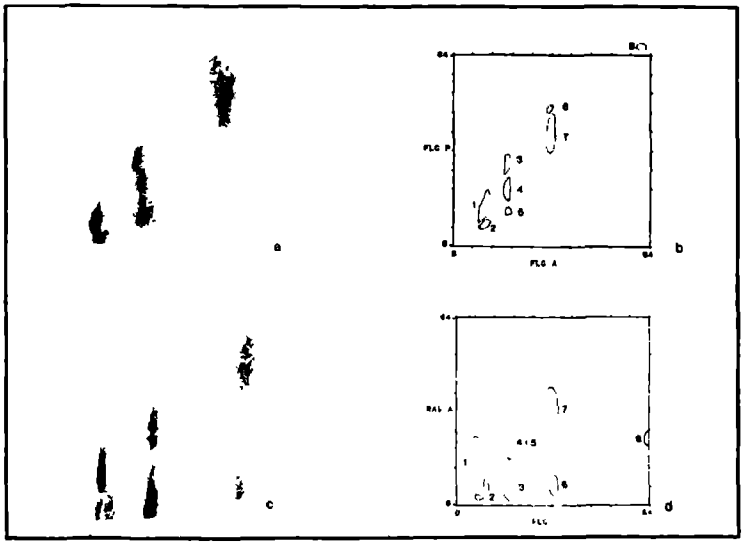


FIG. (5.2) *a) Two-parameter FCM analysis of a cell suspension preparea from rat testis: peak amplitude of the fluorescence signal (ordinate) versus its total intensity integrated over time (abscissa). Two subpopulations are distinquished within the haploid (DI=0.5) and tetraploid (DI=2.0) regions, whereas in the diploid (DI=1.0) region three subpopulations are seen. b) Schematic drawing of the eight subpopulations shown in the scattergram of Fig. 5.2a. Ordinate: The peak amplitude of the fluorescence signal. Abscissa: Total intensity integrated over time. c) Two-parameter FCM analysis of rat testis cells using right-angle light scatter (ordinate) versus total intensity of the signal over time (abscissa). An improved separation of the two subpopulations in the 4c (DI=2.0) region can be observed, which facilitates the sorting, identification and quantitation of each subpopulation d) Schematic drawing of the scattergram shown in Fig. 5.2b. Ordinate: right angle scatter; abscissa: the total intensity of the signal over time.*

Fig. 5.6 shows the cytological documentation of the different sorted populations.

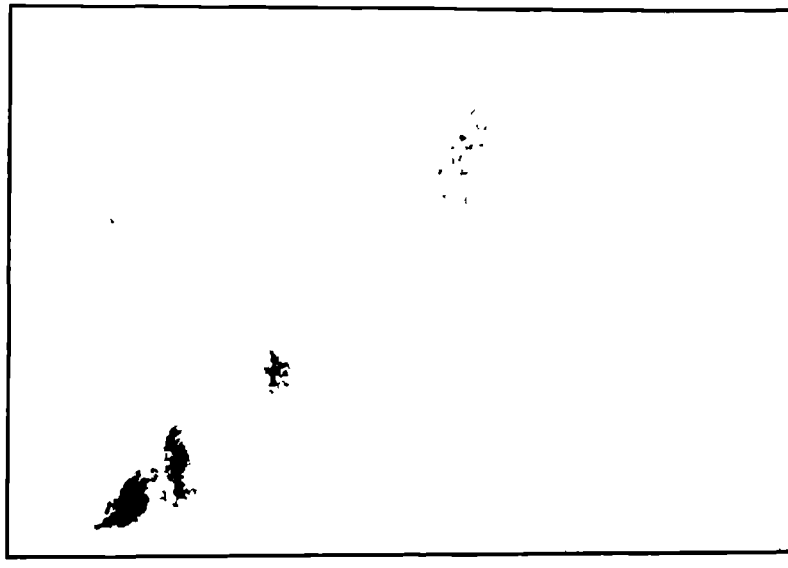


FIG. (5.3) *DNA fluorescence distribution from a single cell suspension of a rat testis using a 128x128 data field. Abscissa: Total fluorescence intensity; ordinate: number of cells, mithramycin stain, number of measured cells: 50,000.*

DISCUSSION

The present study uses two DNA-derived FCM parameters in place of one to analyze the cell populations present in rat testis. The total cellular fluorescence integrated over time gives an accurate determination of total stainable DNA per cell. This is seen in Figs. 5.1 and 5.2 and has been amply documented in the past (14). Differences in the state of chromatin condensation may be reflected in this total DNA parameter (10), and these differences as well as shape differences are partially responsible for the distinction between round and elongated spermatids seen in Fig. 5.1. The one-parameter histogram does not show a good resolution in the 1c (DI=0.5) region. In Fig. 5.4 the 6:1 ratio of round to elongated

Compartment.	Sorted cell type	Total population %
(*) 1	Round spermatids	59.5 ±3.1
2	Elongated spermatids	10.5 ±3.4
3	Leydig and Sertoli cells, fibroblasts	2.8 ±0.3
4	Secondary and preleptotene spermatocytes some spermatogonia	7.5 ±0.5
5	Spermatogonia and clusters of spermatids	4.8 ±1.5
6	Leptotene and zygotene spermatocytes and some spermatogonia	1.4 ±0.3
7	Pachytene primary spermatocytes	6.4 ±1.3
8	Spermatocytes (?)	1.0 ±0.4

FIG. (5.4) *Identification of the sorted cells and quantitation of the different compartments demonstrated by two-parameter DNA flow cytometry.* (*) *See Fig. 5.2b for the definition of the compartments.*

spermatids is inconsistent with the approximately equal numbers of these cells in the testis. Much better discrimination of round and elongated spermatids may be performed using other preparation protocols (6,15).

The shape of the fluorescence signal is influenced by the nuclear size such that for equivalent amounts of stainable DNA (equivalent integrated DNA signals), larger nuclei have longer, lower fluorescence signals, while smaller, more condensed nuclei produce shorter, higher peaks. Since testicular germ cell nuclei vary considerably in size and shape during the various stages of maturation, use of the height of the DNA fluorescence signal as a second parameter produces useful information in FCM analysis of testis cells. For example, the peak in the DNA index region 1.0 in the histograms represents spermatogonia, preleptotene spermatocytes, secondary spermatocytes and non-cycling cells (Leydig cells, Sertoli

FIG. (5.5) *Photomicrograph of an octaploid cell. Note the highly condensed chromatin structure. Magnification: x1000. Haematoxylin stain.*

cells, and the like). This region thus constitutes a very heterogeneous mixture of cell types for which a single-parameter histogram has little interpretative value. Using two-parameter DNA analysis, non-germ cells can be distinguished from germ cells in the 2c DNA (DI=1.0) region (Fig. 5.2). This distinction allows separate analysis of pharmacologic effects on germ cells and other cellular components of normal testis, as well as on the various subsets of germ cells. Thus, two-parameter DNA-derived FCM analysis of testis cell subpopulations can provide sufficient resolution to recognize important effects of drugs and other manipulations on components of spermatogenesis (van Kroonenburgh et al, in preparation).

Differences in condensation and arrangement of chromatin during maturation of spermatids allows demonstration of the two distinct peaks of DNA fluorescence instead of one broad one in the DNA index region 0.5 (4,9). The binucleated cells found in the DNA index region 1.0 (Fig. 5.2) are probably secondary spermatocytes. Compartment 8 reveals information that could not be obtained from histological slides. We hypothesize that these characteristic

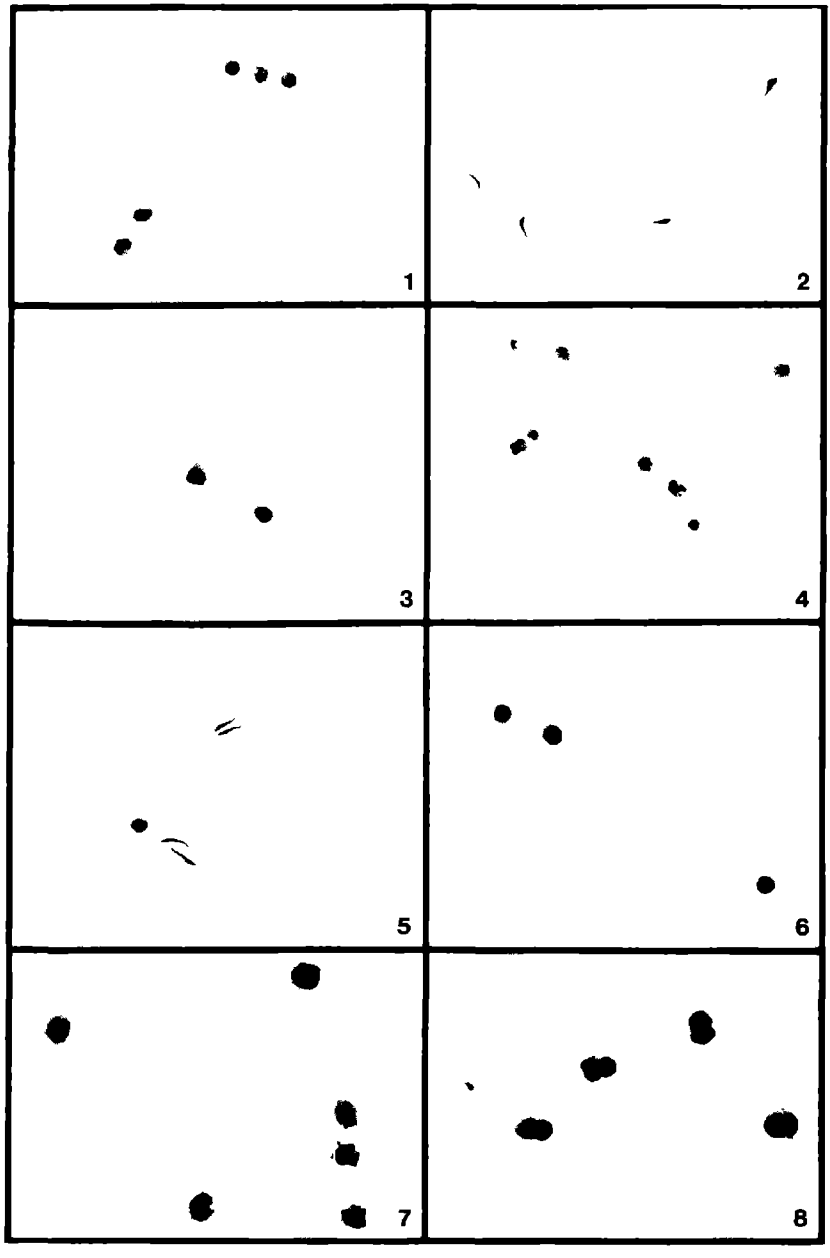


FIG. (5.6) *Photomicrograph of cells sorted from the eight subpopulations (see text). The numbers of the photomicrographs correspond with the number of the population as described in Fig. 5.2. Original magnification for all photomicrographs: x160. Haematoxylin stain.*

binucleated cells (Figs. 5.5, 5.6) are not artefacts (7), but cells normally present in rat testis as 1.0% of the total population. These 8c (DI=4.0) cells were also observed in mouse testis by Dr. U. Hacker, Münster, Germany (unpublished results 1983). Whether these cells belong to the germ cells (primary spermatocytes) or non-germ cells remains to be investigated. It can be concluded that two-parameter flow cytometry can be used as a more sensitive analytical tool in observing effects on spermatogenesis and can give more detailed information as compared to single-parameter DNA flow cytometry.

ACKNOWLEDGEMENTS

It is a pleasure to thank Mr. J. Koedam and Mr. H. van Wezel for skilful technical assistance.

References

1. Bellvé AR, Milette CF, Bhatnagar YM and O'Brien DA: Dissociation of the mouse testis and characterization of isolated spermatogenic cells. J. Histochem. Cytochem. 7, 480-494, 1977.

2. Clausen OPF, Parvinen M, Kirkhus B: Stage-related variations in DNA fluorescence distribution during rat spermatogenic cycle measured by flow cytometry. Cytometry 6, 421-425, 1982.

3. Clausen OPF, Purvis K, Hansson V: Assesment by microflow fluorometry of the purity of interstitial cell suspensions from the rat testis. J. Reprod. Fert. 52, 387-390, 1978.

4. Clausen OPF, Purvis K, Hansson V: Application of Micro-flow fluorometry to studies of meiosis in the male rat. Biol. Reprod. 17, 555-560, 1977.

5. Clermont Y: Quantitative analysis of spermatogenesis of the rat: A revised model for the renewal of spermatogonia. Am. J. Anat. 111, 111-129, 1962.

6. Evenson DP, Melamed MR: Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J. Histochem. Cytochem. 31, 248-253, 1983.

7. Grogan WM, Farnham WF, Sabau JM: DNA analysis and sorting of viable mouse testis cells. J. Histochem. Cytochem. 29, 738-746, 1981.

8. Hacker U, Schuman J, Göhde W: Effects of acute γ-irradiation on spermatogenesis as revealed by flow cytometry. Acta Radiol. Oncol. 19, 361-368, 1980.

9. Hacker U, Dissertation: Biologische dosimetrie ionisierender strahlung am modell der spermatogenese der Maus. Munster, W. Germany, 1981.

10. Hamilton VT, Habbersett MC, Herman CJ: Flow microfluorometric analysis of cellular DNA: critical comparison of mithramycin and propidium iodide. J. Histochem. Cytochem. 28, 1125-1128, 1980.

11. Libbus BL, Schuetz AW. Analysis of the progression of meiosis in dispersed rat testicular cells by flow cytofluorometry. Cell Tiss. Kinet. 11, 377-391, 1978.

12. Meistrich ML, Lake S, Steinmetz LL, Gledhill BL: Flow Cytometry of DNA in mouse sperm and testis nuclei. Mut. Res. 49, 383-396, 1978.

13. Pfitzer P, Gilbert P, Rolz G, Vyska K: Flow cytometry of human testiticular tissue. Cytometry 3: 116-122, 1982.

14. Tannenbaum E, Cassidy M, Alabaster O, Herman C: Measurement of cellular DNA mass by flow microfluorometry using a biological internal standard. J. Histochem. Cytochem. 26, 145-148, 1978.

15. Zante J, Schumann J, Barlogie B, Gohde W, Buchner T: New preparation and staining procedures for specific and rapid analysis of DNA distributions· 2nd International Symposium on Pulse Cytophotometry, Munster, ed. by W. Gohde, Schumann J, Buchner T. European Press, Ghent, 97-106, 1976.

16. Zante J, Schumann J, Gohde W, Hacker U: DNA-fluorometry of mammalian sperm. Histochem. 54, 1-7, 1977.

17. Zimmermann A, Truss FT: The prognostic power of flow-through cytophotometric DNA determinations for testicular diseases. Analyt. Quant. Cytol. 2, 247-251, 1980.

CHAPTER 6

EFFECTS OF TESTICULAR IRRADIATION ON STEM CELL SURVIVAL, HORMONAL ENVIRONMENT AND SPERMATOGENIC CELLS IN WISTAR RATS

M.J.P.G. van Kroonenburgh, W.A.J. van Daal,
J.L. Beck, H.M. Vemer, R. Rolland and C.J. Herman

(Submitted for publication in Acta Radiologica Oncology)

ABSTRACT. Adult male rats were exposed to 3.0 Gy of local testicular irradiation. Testes of irradiated and non-irradiated rats were examined histologically and flow cytometrically, at several intervals up to 78 days after irradiation. Concentrations of FSH, LH and testosterone determined at these intervals were not different from those of controls. The survival of stem cells were measured 11 weeks after irradiation (with doses varying from 1.0 Gy to 6.0 Gy at 0.6 Gy/minute) by means of the repopulation index and by the number of haploid cells (spermatids). Correlation between both methods and relation to stem cell survival were discussed. The dose response curves yielded Do values of stem cell survival of 2.33 ± 0.06 Gy (repopulation index) and 2.08 ± 0.08 Gy (number of haploid cells). The Do value of the rat was not much different from that found in mice. It was concluded that the used parameters can offer us insight when studying hormonal substances during irradiation.

INTRODUCTION

With the success of irradiation in prolonging the lives of patients with malignant diseases one started to realize that permanent gonadal damage may be induced. A treatment which could reduce the magnitude of testicular damage is highly desirable since it may result in preservation of reproductive function.

It is known (7,8,15,28) that radioresistant stem cells play a crucial role in long term effects of irradiation on fertility. Shortly after testicular irradiation stem cell spermatogonia are almost the only spermatogonia present (1,9). It was suggested (26,28), that the sharp increase of stem cell radiosensitivity occuring shortly after irradiation, could be attributed to a change of surviving stem cells towards a much more radiosensitive phase of their cell cycle. From a theoretical point of view, hormones could be considered to be able to reduce or to avoid radiation damage, arresting stem cell division in the radioresistant phase, resulting in preservation of reproductive function. While proof of this hypothesis is lacking, its validity can be tested by interrupting the pituitary-gonadal axis by hormones during testicular irradiation. There are a number of studies which have set precedent in terms of protection of spermatogenesis by hormones (6,10,11,16,21,24). However, these studies have in general concentrated upon protection from cytotoxic drugs.

Before studying if hormonal treatment during irradiation is a possible mechanism to protect the testis from radiation damage, definition of parameters are necessary. The purpose of this investigation was to define the radiosensitivity of stem cells and to study the effects of irradiation on hormonal, histological, and flow-cytometrical parameters.

MATERIALS AND METHODS

The experiments were started with 3-months-old male outbred Wistar rats (Cpb:WU) each weighing about 300 g. The animals were allowed free access to dry pellet food (RMH-TM HOPE-FARMS, Woerden,

The Netherlands) and tap water. They were housed in groups of 4 in well ventilated cages at 22°C with a day/night rhythm of 12 hours. 24 rats were used to measure the Do value of stem cell survival. Groups of 4 rats each were irradiated with 0 (controls), 1.0, 2.0, 3.0, 4.0 and 6.0 Gy and killed 11 weeks after irradiation. 36 Wistar rats were used to study the local effect of 3.0 Gy testicular irradiation dose and were divided into two groups consisting of 18 rats each. Group 1 was given a single dose of testicular irradiation dose of 3.0 Gy, whereas Group 2 received the same procedure except no irradiation was given (controls). Serum hormone concentrations, number of tetraploid and haploid cells and testicular histology were quantified at day 0, 7, 14, 21, 28, 39, 52, 65, 78 after irradiation. Blood was collected by heart puncture and after centrifugation (1200g, 10 min) serum samples were stored at -20°C until analysed. The testes were removed and weighed immediately after heart puncture.

Irradiation conditions

The rats were anaestized with an Alyrane (2-chloor-1,1,2-trifluorethyl-difluor methylether) oxygen gas mixture and irradiated in prone position with the 18 MV photon beam of a Saturne linear accelerator (CGR, BUC, France). The Saturne is equipped with a mercury shielded irregular field system device (2) which was used to define 4 irradiation portals of 4x5 cm each, so that 4 rats could be irradiated on their scrotum simultaneously. To achieve a dose homogeniety in the target volume within 10% it was necessary to get rid of the build region in the beam. Therefore a 1.5 cm thick plexiglas plate was positioned just above the rats. The dose rate during the experiments was 0.6 Gy/minute.

Stemcell survival assays

Spermatogonial stem cell survival was evaluated by the repopulation index (RI) and by counting the number of haploid cells

(spermatids) by using flow cytometry. The left testis from each killed animal was fixed in Bouin's fluid and embedded in paraffin wax. From each testis three sections, separated from each other by a distance of 500 µm, were taken from the midportion of the testis and stained with Periodicacid-Schiff (PAS). The percentage of tubulus which showed spermatogenic repopulation in histological sections, 11 weeks after irradiation, was defined as the the repopulation index (25). A tubules was considered to repopulate when it contained at least one spermatogonium. The right testis was used for flow cytometric analysis. Radiosensivity can be estimated by the dose required to reduce survival to 37% of the initial value on the straight-line portion of a survival curve (Do value). The Do value of the curve was calculated with a linear regression model by a simple least-square analysis at the mean RI values as was previously described (25).

Histology

The left testis from each animal was fixed in Bouin's fluid, dehydrated in 70% ethanol and embedded in paraffin wax. Duplicate transverse sections of 5µm were taken from the midportion of the testis and stained with Periodicacid-Schiff (PAS) with and without prior diastase digestion. For histological measurement, the germ cell maturation sequence of 14 stages as proposed by Leblond and Clermont (20), was used. The counts were made on cross sections of 25 seminiferous tubules. The numbers of spermatogonia or preleptotene spermatocytes were expressed as numbers per 100 Sertoli cells. Expressing the data in this way corrects for tissue shrinkage and permits a comparison of germ cell counts from one time interval to another. Spermatogonia of types Ao and A1 were scored as a single class of cells since in our material they could not be differentiated. Type B spermatogonia were scored at stage 6; intermediate spermatogonia at stage 3 and preleptotene spermatocytes at stage 8 of the cycle.

Flow cytometry

The right testis from each animal was dissected free from fat and connective tissue. To obtain a fluorescent stained single cell suspension, preparation and staining procedure was caried out as previously described (18). Haploid and tetraploid cells were measured by flow cytometric analysis which was performed on an Ortho System 50-H (Ortho Instruments; Westwood, MA, U.S.A) using the 457 nm line of the argon ion laser (Spectra Physics, Mountain View, CA, U.S.A) at 200 mW power.

Hormone measurements

Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double-antibody solid phase radioimmunoassays using the rat gonadotrophin kit reagents supplied by NIADDK, Bethesda, MD, U.S.A. The measurements were expressed as µg/l in terms of the NIADDK-rat FSH-RP-1 and LH-RP-1 standards, respectively. The minimum detectable concentrations were 10 µg LH/l and 50 µg FSH/l. The precision was 5.9% within assays and 6.7% between assays for duplicate measurements of FSH in a serum pool (mean value 264 µg/l) in 15 consecutive assays. For LH these values were, respectively, 3.9% and 9.6% (mean: 59 µg/l) in 15 consecutive assays. Testosterone was measured by a dextran-coated charcoal radioimmunoassay after extraction of serum samples with diethyl ether. The antiserum used was raised in a rabbit and directed against Testosterone-3-(O-carboxymethyl)-oxime-BSA. The sensitivity of the assay was 0.1 nmol/l and the precision, as calculated from a serum pool (mean value: 1.85 nmol/l) after 14 consecutive measurements), was 3.9% within assays and 5.7% between assays.

Statistical analysis was performed by using the two-side Mann-Whitney U test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

A semilogarithmic plot of the percentage of stem cell survival versus radiation exposure was determined from the RI (Fig. 6.1) and from the number of haploid cells (Fig. 6.2). In view of the better multiple correlation coefficient, the data point of 1.0 Gy was excluded. The regression analysis, without the 1.0 Gy data points yielded (mean ± s.d.) Do values of 2.33 ± 0.06 Gy (RI) and 2.08 ± 0.08 Gy (haploid cells). The logarithm of the RI was a linear function of dose (multiple correlation coefficient >.95). The data points of both methods (expressed as fractions of controls) were not significant ($P<0.05$) different from each other. The n value (extrapolation number) was 167 (RI) and 2.12 (number of haploids as

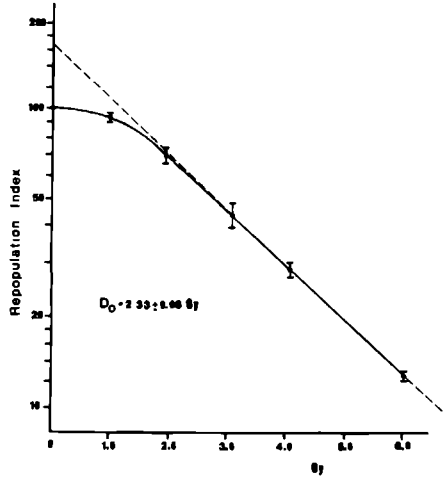


FIG. (6.1) *Stem cell survival as measured by the repopulation index, 11 weeks after irradiation with different dosages. Values are mean ± s.d. of 4 rats.*

a fraction of controls).

The effect of 3.0 Gy single dose irradiation on testes weight, the number of spermatogonia and preleptotene spermatocytes and on the serum concentrations of FSH, LH and testosterone are presented in Fig 6 3. Testes weight declined to 50% of those of control value at day 39 As a result of this irradiation dose a large fraction of type A, intermediate and Type B spermatogonia and

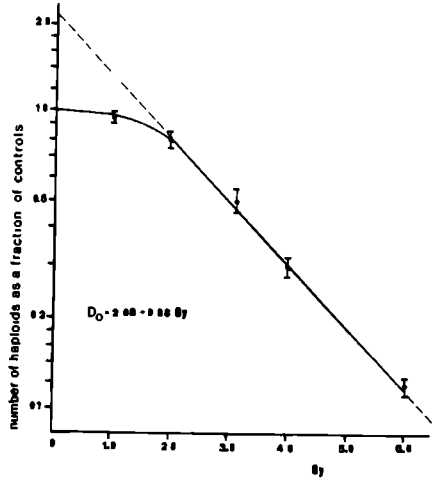
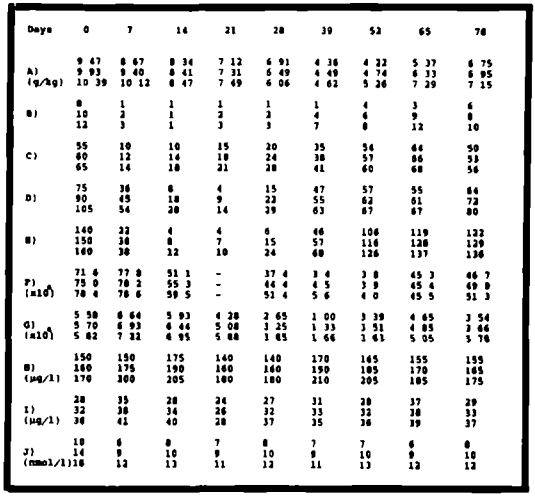


FIG. (6.2) *Stem cell survival as measured by the number of haploid cells, 11 weeks after irradiation with different dosages. Values are mean ± s.d. of 4 rats.*

preleptotene spermatocytes were rapidly destructed (Fig. 6 3).

Recovery was seen from day 14 on. The numbers of type intermediate spermatogonia showed an overshoot phenomenon. The elimination of spermatogonia resulted in a progressive disappearance of the numbers of tetraploid and haploid cells (Fig. 6.3). The total number of spermatids dropped to 10% of the control value. It can be seen (Fig. 6.3), that recovery of spermatids after 3.0 Gy local irradiation commenced after 52 days. The concentrations of FSH, LH and testosterone were equal to that of controls at each time interval (Fig. 6.3).



Days	0	7	14	21	28	39	52	65	78
A)	9 47	8 67	8 34	7 12	6 91	4 38	4 32	5 37	6 75
	9 93	9 40	8 41	7 31	6 49	4 49	4 74	6 33	6 95
(g/kg)	10 39	10 12	8 47	7 49	6 06	4 62	5 26	7 39	7 15
B)	0	1	1	1	1	1	4	3	6
	10	2	1	2	3	4	6	9	8
	12	3	1	3	3	7	8	12	10
C)	55	10	10	15	20	35	54	44	50
	60	12	14	18	24	38	57	66	53
	65	14	18	21	28	41	60	68	56
D)	75	36	8	4	15	47	57	55	64
	90	45	18	9	22	55	62	61	72
	105	54	28	14	29	63	67	67	80
E)	140	33	4	4	6	46	106	119	122
	150	38	6	7	15	57	116	128	129
	160	38	12	10	24	68	126	137	136
F) ($\times 10^6$)	71 6	77 8	51 1	-	37 4	3 4	3 8	45 3	46 7
	75 0	78 2	55 3	-	44 4	4 5	3 9	45 4	49 9
	78 4	78 6	59 5	-	51 4	5 6	4 0	45 5	51 3
G) ($\times 10^6$)	5 58	6 64	5 93	4 28	2 65	1 00	3 39	4 65	3 54
	5 70	6 93	6 44	5 08	3 25	1 33	3 51	4 85	3 66
	5 82	7 32	6 95	5 88	3 85	1 66	3 61	5 05	5 76
H)	150	150	175	140	140	170	165	155	155
	160	175	190	160	160	190	185	170	165
(µg/l)	170	300	205	180	180	210	205	185	175
I)	28	35	28	24	27	31	28	37	29
	32	38	34	26	32	33	32	38	33
(µg/l)	36	41	40	28	37	35	36	39	37
J)	10	6	8	7	8	7	7	6	8
	14	9	10	9	10	9	10	9	10
(nmol/l)	18	12	13	11	12	11	13	12	12

FIG. (6.3) *Effect of a single dose irradiation of 3.0 Gy on (mean ± range) testicular weight (A), on the numbers per 100 Sertoli cells of type A (B), Intermediate (C) and type B spermatogonia (D) and preleptotene spermatocytes (E), on the number of tetraploid cells (F; mainly pachytene spermatocytes), on the number of haploid cells (G; spermatids) and on the serum hormone concentrations of FSH (H), LH (I) and testosterone (J) in adult rats.*

DISCUSSION

Two assays for testicular stem cell survival have been aplied to obtain survival curves. The number of clones in the testis found several weeks after irradiation have been described (25,29) as a direct measurement of stem cell survival. Because there is also a quantitative correlation (22) between testicular stem cell survival and sperm production and since haploid cells are corresponding only to spermatids and to no other testicular cells, the number of haploid cells as measured by flow cytometry can also be used as a measurement of stem cell survival (12). The relationship between spermatid production and stem cell number may be linear. However,

for the mouse, Meistrich (22) has demonstrated that although sperm production and stem cell survival as measured by RI were highly correlated the relationship was not linear (the regression line had a slope of 1.7 on a log versus log plot). The evaluation of the radiosensitivity of stem cells shows a good parallel between both stem cell assays in the actual dose range. Our results are, however, in contrast with those of others. Clow and Gilette (3) and Erickson (8), found Do values of 3.80 Gy and 3.73 Gy, respectively. A comparison of the reported Do values for mice in literature by Ruiter-Bootsma et al. (26) and Withers et al. (28), indicates that our Do value of the rat is not much different from that found in the mouse (2.4 Gy and 1.8 Gy). This is in accordance with the fact that the dose required to produce infertility (13,17) in the mouse is the same as that in the rat. However, in the low dose range when the repoplation index exceeds the value of 0.5, the assay reliability is controversial as multicellular origin of colonies can not be excluded. Correction must therefore be made for the possibility of clone confluence (23). If so, the corrected mean Do value of the RI values was even lower (1.8 Gy).

Since spermatogonia are so very radiosensitive (1,7), a maturation depletion occurs of other spermatogenic cells in their order of developmental sequence, eventually resulting in loss of sperm. This is clearly shown by the decrease in number of preleptotene and pachytene spermatocytes (the numbers of tetraploid cells are mainly consisting of pachytene spermatocytes (19)) and by the low numbers of spermatids (number of haploid cells) from day 39 till day 52. This decrease in the number of pachytene spermatocytes and spermatids was not parallelled by an alteration in serum hormone concentrations Studies (5,14) showed an increase in serum FSH depending on the used dose of irradiation. Conflicting results have, however, been reported after a irradiation dose of 3.0 Gy. Cunningham and Huckins (4) and Verjans and Eik-Nes (27) showed no alteration in FSH levels, whereas Hopkinson et al (14) did. Our findings agree with the findings of Cunningham and Huckins (4), that the selective abscence of spermatogonia and spermatocytes do not affect serum hormone concentrations. From our results we conclude that the used parameters offers us insight when studying hormonal

substances during irradiation. This will be the subject of a subsequent report.

ACKNOWLEDGEMENTS

We acknowledge the skilful technical assistance of Mr J. Koedam and H. van Wezel; of Dr H.J. van Gasteren for advice and help; of Dr S. Raiti of NIADDK, Bethesda, MD, U.S.A. for the gift of the LH and FSH reagents; of Dr C.M.G. Thomas and Dr M.F.G. Segers, Dept. of Obstetrics and Gynaecology for measuring FSH, LH and testosterone levels in serum and we acknowledge Dr J.W. Scholtz for histological counting.

References

1. Bianchi M: Cytotoxic insult to germinal tissue. In: Potten CS, Hendry JH (eds). Cytotoxic insult to tissue. Churchill Livingstone, Edinburgh, 1983.

2. Brouwer WFM, Kazem I and van Mil CJHM: Four years experience with a mercury shielded irregular field system (MSIFS). Int. J. Radiat. Oncol. Biol. Phys. 10, 225-229, 1984.

3. Clow DJ and Gilette EL: Survival of type A spermatogonia following X-irradiation. Radiat. Res. 42, 397-404, 1970.

4. Cunningham GR and Huckins C: Serum FSH, LH and Testosterone in Co^{60} γ irradiated male rats. Radiat. Res. 76, 331-338, 1978.

5. Delic JI, Hendry JM, Morris ID and Shalet SM: Testicular endocrine injury in the adult rat after X-irradiation: Dose and time relationships. Proceedings of the seventh international congress of radiation research (eds: Broerse JJ et al.; Martinus Nijhoff Publishers) Amsterdam C2-03 1983.

6. Delic JI, Bush C and Peckham MJ: Protection from procarbazine-induced damage of spermatogenesis in the rat by androgen. Canc. Res. 46, 1909-1914, 1986.

7. Dym M and Clermont Y: Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. Am. J. Anat. 128, 265-282, 1970.

8. Erickson BH: Effect of Co^{60}-γ-radiation in the stem and differentiating spermatogonia of the post pubertal rat. Radiat. Res. 68, 433-448, 1976.

9. Erickson BH: Survival and renewal of murine stem spermatogonia following Co^{60}-γ-radiation. Radiat. Res. 86, 34-51, 1981.

10. Glode LM, Robinson J, Gould SF, Nett TM and Merril D: Protection of spermatogenesis during chemotherapy. Drugs Exptl. Clin. Res. 7, 367-378, 1980.

11. Glode LM, Robinson J, Gould SF: Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropin-releasing hormone. Lancet. I, 1132-1134, 1981.

12. Hacker-Klom U: Long term effects of ionizing radiation on mouse spermatogensis. Acta Radiol. Oncol. 24, 363-367, 1985.

13. Hertwig P: Unterscheide in der Entwicklungsfahigkeit von F1 Mausen nach Röntgenbestrahlung von Spermatogonien, fertigen und unfertigen Spermatozoen. Biol. Zentrabl. 58, 273-301, 1938.

14. Hopkinson CRN, Dulisch B, Gauss G, Hilscher W and Hirschlauser C: The effect of local testicular irradiation on testicular histology and plasma hormone levels in the male rat. Acta Endocr. 87, 413-423, 1978.

15. Huckins C: Behaviour of stemcell spermatogonia in the adult rat irradiated testes. Biol. Reprod. 19, 747-760, 1978.

16. Johnson DH, Linde R, Hainsworth JD, Vale W, Rivier J, Stein R, Flexner J, van Welch R and Greco A: Effect of a luteinizing hormone releasing hormone agonist given during combination chemotherapy on posttherapy fertility in male patients with lymphoma: Preliminary observations. Blood. 65, 832-836, 1985.

17. Jones EA: Number of spermatogonia after X-irradiation of the adult rat. Int. J. Radiat. Biol. 2, 157-170, 1960.

18. Kroonenburgh van MJ, Beck JL, Scholtz JW, Hacker-Klom U and Herman CJ: DNA analysis and sorting of rat testis cells using two-parameter flow cytometry. Cytometry 6, 321-326, 1985.

19. Kroonenburgh van MJ, Beck JL, van Gasteren J and Herman CJ: A computer model of spermatogenesis in the rat; Correlation of autoradiographical data with flow cytometry. Cell Tiss. Kinet., In Press, 1986.

20. Leblond CP and Clermont Y: Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55, 548-573, 1952.

21. Lewis RW, Dowling KJ and Schally AV: D-Tryptophan-6 analog of luteinizing hormone-releasing hormone as a protective agent against testicular damage caused by cyclophosphamide in baboon.

Proc. Natl. Acad. Sci. USA 82, 2975-2979, 1985.

22. Meistrich ML: Quantitative correlation between testicular stem cell survival, sperm production, and fertility in the mouse after treatment with different cytotoxic agents. J. Androl. 3, 58-68, 1982.

23. Meistrich ML, Finch M, Lu CC, de Ruiter-Bootsma AL and de Rooij DG: Strain differences in the response of mouse testicular stem cells to fractionated radiation. Radiat. Res. 97, 478-487, 1984.

24. Nseyo HO, Huben RP, Klioze SS and Pontes JE: Protection of germinal epithelium with luteinizing hormone-releasing hormone analogue. J. Urol. 134, 187-190, 1985.

25. Ruiter-Bootsma AL, de Kramer MF and de Rooij DG: Response of stem cells in the mouse testis to fission neutrons of 1 MeV mean energy and 300 KV X-rays. Methodolgy, dose response studies, relative biological effectiveness. Radiat. Res. 67, 56-68, 1976.

26. Ruiter-Bootsma AL, de Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1 MeV mean energy of 300 KV X-rays. Radiat. Res. 71, 579-592, 1977.

27. Verjans HL and Eik-Nes KB: Hypothalamic-pituitary-testicular system following testicular X-irradiation. Acta Endocrinol. 83, 190-200, 1976.

28. Withers HR, Hunter N, Barkley HT and Reid BO: Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testes. Radiat. Res. 57, 88-103, 1974.

29. Withers HR: Spermatogenic colonies. In: Potten CS, Hendry JH (eds) Cell clones. Manual of mammalian cell techniques. Churchill Livingstone, Edinburgh, 1985.

CHAPTER 7

EFFECTS OF DANAZOL ON SPERMATOGENESIS IN ADULT RATS

M.J.P.G. van Kroonenburgh, J.L. Beck, H.M. Vemer,
C.M.G. Thomas, R. Rolland and C.J. Herman

(Published in Journal of Reproduction and Fertility 77,233-238, 1986)

ABSTRACT. Adult male Wistar rats were treated with Danazol (4 mg/day s.c.) for 52 days. The drug produced a marked, rapid drop in serum testosterone concentrations to very low levels and caused a slower decrease in serum FSH, LH and testis weight. Flow cytometric analysis of testicular cell suspensions showed a decline in the absolute numbers of haploid cells (spermatids), tetraploid cells (mainly pachytene spermatocytes) and of cells in the S-phase of the division cycle, suggesting that Danazol inhibited proliferation of spermatogonia and/or primary spermatocytes. Histological counting of the different types of spermatogonia, however, revealed no significant change in their numbers during Danazol treatment. It is concluded that Danazol inhibited spermatogenesis primarily after the preleptotene stage of primary spermatocytes.

INTRODUCTION

Danazol (a 2,3-isoxazol derivative of 17-α-ethinyl-testosterone) has been described as an active pituitary gonadotrophin inhibitory agent, suppressing the pituitary-gonadal axis in animals (2,3,4,5,11,12) and in man (10,14,15,17). Danazol appeared to suppress spermatogenesis by depletion of spermatocytes, spermatids and spermatozoa (14), but the stage or stages of spermatogenesis which are affected have not yet been defined. Ulstein et al. (17) suggested that Danazol acts by inhibiting spermatogenesis at the spermatocyte/spermatid level. In most studies (3,5,11), animals without functional gonads or immature rats were used to assess the antigonadotrophic properties of Danazol. According to Barbieri et al. (1), these studies are not directly applicable to animals with intact gonadal function. At all tested doses (1.25-10 mg/kg s.c.) of Danazol in male rat, serum LH and FSH concentrations were unsuppressed, whereas the suppression of serum testosterone appeared to be dose-dependent (1).

The purposes of the present investigation were (1) to study the antigonadotrophic effects of Danazol in intact mature male rats by measuring serum gonadotrophin concentrations, and (2) to examine the effect on the different steps of spermatogenesis by counting the different types of spermatogonia. In earlier published reports (3,5,11) Danazol had antigonadotrophic effects in rats at a dosage of 4 mg s.c. given daily, and so this dose was used throughout the present study.

MATERIALS AND METHODS

The experiments were started with 3-month-old male outbred Wistar rats (Cpb:WU) with a body weight of about 300 g. The animals were allowed free access to dry pellet food (RMH-TM HOPE-Farms, Woerden, The Netherlands) and tap water. They were housed in groups of 4 in well ventilated cages at 22°C with a day/night rhythm of 12 h. The 42 rats received daily (at 09:00 h) s.c. injections of 4 mg Danazol

(Sterling-Winthrop Research Institute, London, U.K.) suspended in 0.2 ml sesame oil. Groups of 6 rats were killed at 10:00 h under ether anaesthesia before the injection (controls) and at 7, 14, 21, 28, 39 and 52 days after the start of drug injections. Blood was collected by heart puncture and the testes were removed immediately and weighed. After centrifugation (1200 g, 10 min), serum samples were stored at -20°C until analysed.

Hormone measurements

Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double-antibody solid-phase radioimmunoassays using the rat gonadotrophin kit reagents kindly supplied by NIAMDD, Bethesda, Maryland, U.S.A. The measurements were expressed as ng/ml in terms of the NIAMDD-rat FSH-RP-1 and LH-RP-1 standards, respectively. The minimum detectable concentrations were 10 ng LH/ml and 50 ng FSH/ml. The precision was 5.9% within assays and 6.7% between assays for duplicate measurements of FSH in a serum pool (mean: 264 ng/ml in 15 consecutive assays). For LH these values were, respectively, 3.9% and 9.6% (mean: 59 ng/ml in 15 consecutive assays). Testosterone was measured by a dextran-coated charcoal radioimmunoassay after extraction of serum samples with diethyl ether. The antiserum used was raised in a rabbit and directed against testosterone-3-(O-carboxymethyl)-oxime-BSA. The sensitivity of the assay was 0.1 µmol/ml and the precision, as calculated from a serum pool (mean value: 1.85 µmol/ml after 14 consecutive measurements) was 3.9% within assays and 5.7% between assays.

Histology

The left testis from each animal was fixed in Bouin's fluid, dehydrated in 70% ethanol and embedded in paraffin wax. Duplicate transverse sections of 5 µm were taken from the midportion of the testis and stained with periodicacid-Schiff (PAS) with and without

prior diastase digestion. For histological measurement, the germ cell maturation sequence of 14 stages as proposed by Leblond and Clermont (8), was used. The counts were made on cross-sections of 25 seminiferous tubules. The numbers of spermatogonia or preleptotene spermatocytes were expressed as numbers of cells per 100 Sertoli cells. Expressing the data as numbers of cells per 100 Sertoli cells corrected for tissue shrinkage and permitted a comparison of germ cell counts from one time interval to another. Spermatogonia of types Ao and A1 were scored as a single class of cells since in our material they could not be differentiated. Type B spermatogonia were scored at stage 6; intermediate spermatogonia at stage 3 and preleptotene spermatocytes at stage 8 of the cycle.

Flow-cytometry

The right testis from each animal was dissected free from fat and connective tissue. After decapsulation, the tissue was minced carefully with scissors in 15 ml phosphate buffer (pH 7.4; 23.8 mg PO_4^{3-} /ml) and sieved through a 50 µm mesh filter. All preparation steps were done at room temperature. This cell suspension was passed through a 25-gauge, 25 mm needle, centrifuged at 370 g for 10 min and the cell pellet resuspended in 10 ml phosphate buffer by vortexing. Debris was removed by filtration through a nylon filter (pore size: 50 µm). The suspension was again centrifuged at 370 g for 10 minutes and the supernatant discarded. Samples were fixed in 50% ethanol (-20°C) and stored at 4°C until analysed. Staining was carried out as described elsewhere (6,16). Briefly, 1 ml cell suspension (about 3 x 10^6 cells) was centrifuged for 10 min at 370 g and the ethanol wasdiscarded. The cell pellet was resuspended in 1 ml staining solution consisting of 0.1 mg Mithramycin (Serva Fein Biochemica, Heidelberg, W. Germany) per ml 15 mM-$MgCl_2$ in 0.85% NaCl (w/v). Staining was carried out in the dark at room temperature for 30 min. Flow-cytometric analysis was performed on an Ortho System 50-H (Ortho Instruments; Westwood, MA, U.S.A.) using the 457 nm line of the argon ion laser at 200 mW power. A Coulter counter was used to count the number of cells in 1 ml of the cell

suspension. The total cell counts per volume were needed for calculation of the total number of the different cell types in the testis.

Statistical analysis was performed by using the two-sided Mann-Whitney U test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Serum hormone concentrations

There was a significant decrease of serum FSH, LH and testosterone concentration after 7 days of Danazol treatment ($P<0.05$), which persisted throughout the period of Danazol administration (Fig. 7.1). Also a significant ($P<0.05$) decrease in total testicular weight was observed (Fig. 7.2) after 39 days of drug administration.

Germ cell populations

No significant effects of Danazol on the numbers of the different types of spermatogonia or on the numbers of preleptotene primary spermatocytes were detected (Fig. 7.3).

Proliferative activity of testicular cells

An example of a flow-cytometric histogram of untreated testicular cells is presented in Fig. 7.4. The two subpeaks in the 1C region of the histogram represented round (1a) and elongated (1b) spermatids respectively. The geometry of the histograms was symmetrical and distributions were obtained with a coefficient of variation of 3.5% (1C peak).

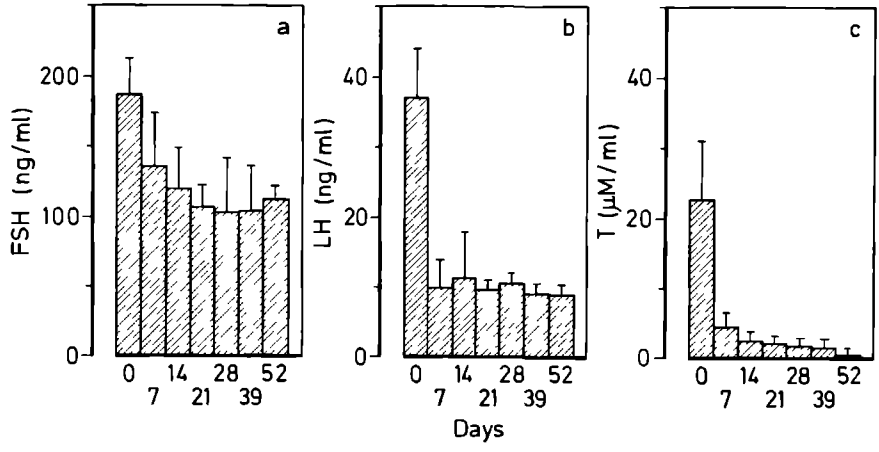


FIG. (7.1) *The effects of Danazol (4 mg/day s.c.) on serum concentrations of (a) FSH, (b) LH and (c) testosterone in adult male rats. Values are mean ± s.d. for 6 rats/group.*

A decrease of total number of haploid cells was observed ($P<0.05$) after 28 days of treatment (Fig. 7.5) and an almost parallel decline of tetraploid cells occurred (Fig. 7.5). A significant decrease ($P<0.05$) of the number of cells in the S-phase (Fig. 7.5) was seen after 39 days of Danazol administration.

DISCUSSION

It is apparent from the present study that Danazol effectively suppresses the pituitary-gonadal axis in adult rats. In our study a significant decrease of gonadotrophins occurred ($P<0.05$) within 7 days of Danazol treatment at a dose of 4 mg/day s.c.. From these data it appears that Danazol treatment of animals with intact gonadal function is accompanied by lowered hormone concentrations (1). Histological counting showed no significant influence of Danazol treatment on the different types of spermatogonia. This supports the evidence that anti-gonadotrophic treatments have

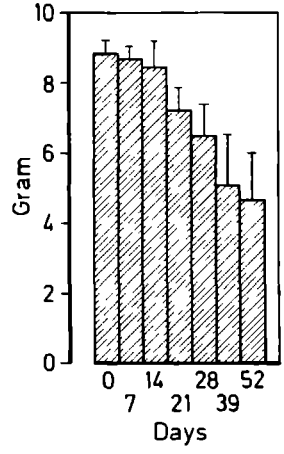


FIG. (7.2) *The effect of Danazol (4 mg/day s.c.) on mean ± s.d. testicular weights of adult rats (6/group).*

similar effects on the specific stages of spermatogenesis (13). Of special interest, however, is the quantitative analysis of changes in proliferative activity of testicular cells in animals receiving Danazol. In normal spermatogenesis the last duplication of DNA takes place in the S-phase of a primary spermatocyte (9). The number of cells in the S-phase therefore reflects the proliferative activity of cells in the early stages of spermatogenesis. From the decrease of cells in the S-phase during Danazol administration (Fig. 7.5), it can be concluded that the drug influenced the proliferative activity of spermatogonia and/or primary spermatocytes, either directly or indirectly. Whether this was due to a decrease in spermatogonia or in preleptotene spermatocytes, or both remains to be investigated. Tetraploid cells consisted mainly of pachytene spermatocytes and only to a lesser degree of spermatogonia in mitosis (7). Finally, during Danazol treatment a drop in total numbers of cells takes place. (Fig. 7.5). The uncoupling of the effects on the total number of cells in the S-phase from effects on gonadotrophin concentrations

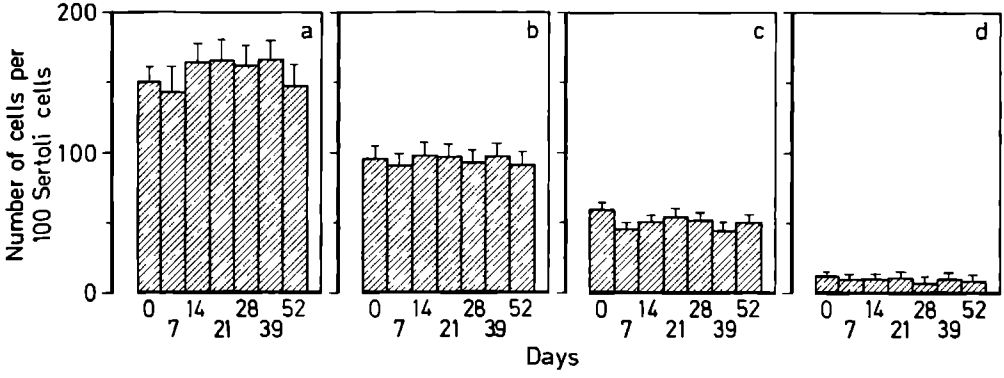


FIG. (7.3) *The numbers (mean ± s.d.) of the different types of spermatogonia, A (d), intermediate (b), and B (c), and preleptotene primary spermatocytes (a) per 100 Sertoli cells.*

suggests that, at least initially, the early stages of spermatogenesis are relatively independent of serum concentrations of FSH, LH and testosterone.

It can be concluded that Danazol suppresses serum concentrations of gonadotrophins in intact adult male rats and inhibits spermatogenesis from the preleptotene stage of the primary spermatocyte onwards. It also has a direct or indirect inhibiting effect on the proliferation of spermatogonia and/or primary spermatocytes.

ACKNOWLEDGEMENTS

We thank Mr J. Koedam for skilful technical assistance; Dr S. Raiti of NIAMDD, Bethesda, Maryland, U.S.A. for the gift of the LH and FSH reagents; Dr M.F.G. Segers for analytical assistance; and Dr J.W. Scholtz for histological counting. Danazol was kindly supplied by Sterling-Winthrop Research Institute, London, U.K. .

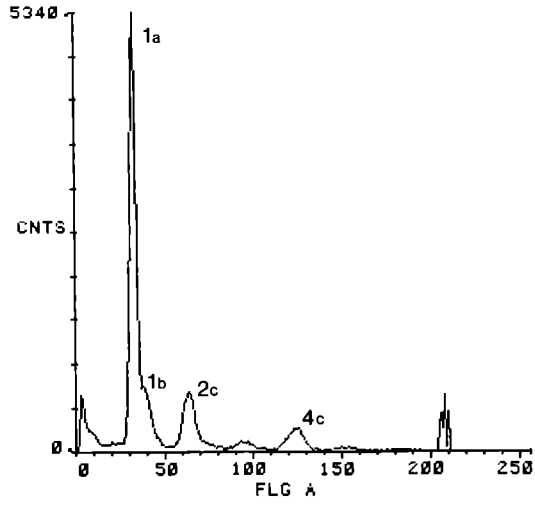


FIG. (7.4) *DNA fluorescence distribution from a single cell suspension of a rat testis. Abscissa: total fluorescence intensity; ordinate: number of cells (counts). The first two peaks (1a,1b) represent haploid cells (round and elongated spermatids respectively), the third (2c) and fourth (4c) peaks represent diploid and tetraploid cells, respectively. Mithramycin stain, Variation coefficient (first peak)= 3.5%.*

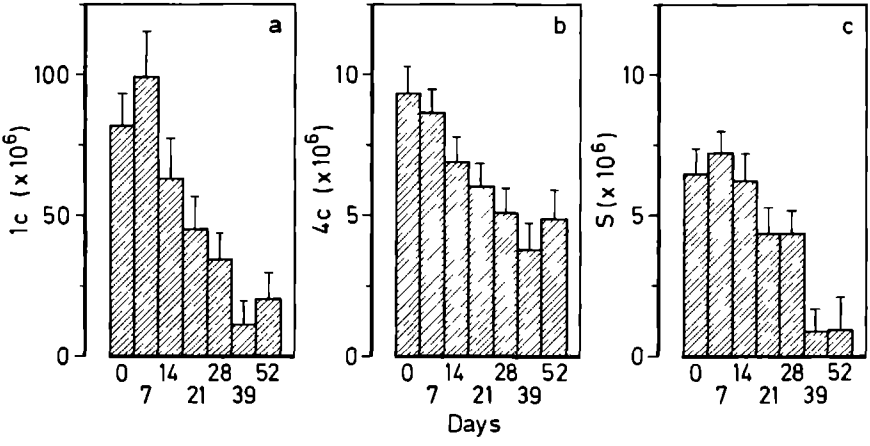


FIG. (7.5) *Mean ± s.d. absolute numbers of (a) haploid (spermatids) cells, (b) tetraploid (mainly pachytene primary spermatocytes) cells and (c) in the S-phase of the division cycle per testis during 52 days of continous treatment with Danazol (4 mg/day s.c.) in adult rats (6/group).*

References

1. Barbieri RL, Caninck JA, Makris A, Todd RB, Davies IJ and Ryan KJ : Danazol inhibits steroidogenesis. Fert. Steril. 28, 809-813, 1977.

2. Dixit VP, Agrawal M and Varma M: Male contraception properties of a new synthetic steroid derivative (Danazol) in Rattus rattus Rufescens. Endokrinology. 77, 30-38, 1981.

3. Dmowski WP, Scholer HFL, Mahesh VB and Greenblatt RB: Danazol a synthetic steroid derivative with interesting physiological properties. Fert. Steril. 22, 9-18, 1971.

4. Dmowski WP: Endocrine properties and clinical application of danazol. Fert. and Ster. 31, 237-251, 1979.

5. Eldridge JC, Dmowski WP and Mahesh VB: Effects of castration of immature rats on serum FSH and LH and of various steroid treatments after castration. Biol. Reprod. 10, 38-446, 1974.

6. Hamilton VT, Habbersett MC and Herman CJ: Flow microfluorometric analysis of cellular DNA: Critical comparison of mithramycin and propidium iodide. J. Histochem. Cytochem. 28, 1125-1128, 1980.

7. Kroonenburgh van MJ, Gasteren van HJ, Beck HJ and Herman CJ: A computer model of spermatogenesis in the rat; correlation with flow cytometric data based on autoradiographic cell cycle properties. (Cell Tiss. Kinet. 19, 171-177, 1986.

8. Leblond CP and Clermont Y: Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N. Y. Acad. Sci. 55, 548-573, 1952.

9. Monesi V: Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis, using tritiated thymidine. J. Cell Biol. 14, 1-18, 1962.

10. Paulsen CA and Leonard JM: Clinical trials in reversible male contraception: I Combination of Danazol plus Testosterone. Regulatory mechanism of male reproductive physiology. ED. Spilman Excerptia Medica Amsterdam, 197-211, 1976.

11. Pedroza E, Vilchez-Martinez JA, Arimura A and Schally AV: Danazol effects on gonadotrophin basal levels and pituitary responsiveness to LH-RH in immature male rats. Contraception. 17, 61-69, 1978.

12. Potts GO, Beyler AL and Schane HP: Pituitary gonadotrophin inhibitory activity of Danazol. Fert. Steril. 25, 367-372, 1974.

13. Russell LD, Malone JP and Karpas SL: Morphological pattern elicited by agents affecting spermatogenesis by disruption of its hormonal stimulation. Tiss. Cell. 13, 369-380, 1981.

14. Sherins RJ, Gandy HM, Thorslund TW and Paulsen CA: Pituitary and testicular function studies. I Experience with a new gonadal inhibitor, 17-α-Pregn-4-en-20 yno-(2,3-d) isoxazol-17-ol (Danazol). J. Clin. Endocr. Metab. 32, 522-531, 1971.

15. Skoglund RD and Paulsen CA: Danazol-testosterone combination: a potentially effective means of reversible male contraception, a preliminary report. Contraception. 7, 357-365, 1973.

16. Tannenbaum E, Cassidy M, Alabaster O and Herman C: Measurement of cellular DNA mass by flow microfluorometry using a biological internal standard. J. Histochem. Cytochem. 26, 145-148, 1978.

17. Ulstein M, Netto N, Leonard J and Paulsen CA: Changes in sperm morphology in normal men treated with Danazol and testoterone. Contraception. 12, 437-444, 1975.

CHAPTER 8

EFFECTS OF A SINGLE INJECTION OF A NEW DEPOT FORMULATION OF AN LH-RELEASING HORMONE AGONIST ON SPERMATOGENESIS IN ADULT RATS

M.J.P.G. van Kroonenburgh, J.L. Beck, H.M. Vemer,
R. Rolland, C.M.G. Thomas and C.J. Herman

(Accepted for publication in the Journal of Endocrinology)

ABSTRACT. Adult male Wistar rats were treated with a single injection (500 µg s.c.) of a new biodegradable depot formulation of the LH-releasing (LHRH) analogue D-Ser(But)^{6}, AzGly^{10}-LH-RH (Zoladex ICI 118630) to evaluate its potential for inhibiting spermatogenesis. The drug produced a marked ($P<0.05$) decrease in serum concentrations of FSH, LH and testosterone with a maximum effect 14 days after treatment. Since striking focal histological changes were seen in the testis after only 1 week, at a time when changes in serum gonadotrophins were minimal, there may be a direct effect of the LHRH analogue on spermatogenesis. Degenerative changes in germ cells as well as Sertoli cells could be observed. Flow cytometric analysis of testicular cell suspensions showed a significant decline in the absolute numbers of haploid cells (spermatids), tetraploid cells (mainly pachytene spermatocytes) and of the numbers of cells in the S-phase of the cell cycle. This suggests that the drug also inhibit proliferation of spermatogonia and/or primary spermatocytes. Testis weight, serum hormone concentrations, and histological and cytological parameters returned to essentially normal values 52 days after the injection. It is concluded that this new method of administration may have practical and pharmacokinetic advantages for the purpose of reversible inhibition of spermatogenesis.

INTRODUCTION

After the discovery of the structure of luteinizing hormone releasing hormone (LHRH) (16) a large series of more potent analogues, were synthesized and investigated (4). Originally, it was thought that these analogues would offer a new therapeutic approach to the treatment of infertility, but it was soon realized (7,12) that they also cause desensitization of the pituitary gland, creating a hypogonadotrophic state. As a consequence, the LHRH analogues were investigated for the purpose of male contraception (15). Recently, the LHRH analogue Zoladex (D-Ser(But)^{6}, AzGly^{10}-LH-RH), has been developed. It is available as a depot formulation, in which the LHRH analogue is dispersed throughout a matrix of d,l-lactide-glycolide co-polymer (9). When injected s.c., the co-polymer slowly degrades and the LHRH analogue is released into the subcutaneous tissue and absorbed into the systemic circulation. This formulation has both practical and pharmacokinetic advantages over daily injections.

The purpose of the present study was to investigate the inhibitory effect of a single s.c. injection of Zoladex depot on spermatogenesis as well as the reversibility of any changes observed. In addition, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone concentrations were measured before and after Zoladex treatment. Counting of spermatogonia and preleptotene spermatocytes in histological sections allowed an assessment to be made of which step(s) in spermatogenesis were affected. Finally, flow cytometric analysis was used to determine the proliferative behaviour of testicular cells after the depot injection.

Animals and treatments

Three-month-old male outbred Wistar rats (Cpb:WU) with a body weight of about 300 g were used. They were allowed unrestricted access to dry pelleted food (RMH-TM HOPE-Farms, Woerden, The Netherlands) and tap water, and housed in groups of four in well-ventilated cages at 22°C with a day/night light cycle of 12. At the beginning of the experiment 42 rats received a s.c. depot of Zoladex (ICI PLC., Pharmaceuticals Division, Macclesfield, Cheshire), this consisted of a cylindrical rod (approximately 3 mm long) containing 500 µg LHRH analogue. Six rats were killed at 10.00 h under ether anaesthesia on days 0 (controls), 7, 14, 21, 28, 39 and 52 after injection of the drug. Blood was collected by heart puncture and the testes were removed immediately and weighed. After centrifugation (1200 g, 10 min) the serum was stored at -20°C until assayed.

Hormone measurements

Serum concentrations of FSH and LH were measured as previously described by Van Geelen et al.(23) by double-antibody solid phase radioimmunoassays using the rat gonadotrophin kit reagents kindly supplied by NIADDK, Bethesda, MD, U.S.A. Measurements are expressed as µg/l in terms of the NIADDK-rat FSH-RP-1 and LH-RP-1 standards, respectively. The minimum detectable concentrations were 10 µg LH/l and 50 µg FSH/l. The precision was 5.9% within assay and 6.7% between assays for duplicate measurements of FSH in a serum pool (mean value 264 µg/l) in 15 consecutive assays. For LH these values were 3.9 and 9.6%, respectively, (mean: 59 µg/l) in 15 consecutive assays. Serum testosterone concentrations were measured by a dextran-coated charcoal radioimmunoassay after extraction with diethyl ether and subsequent isolation of the testosterone fractions by Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden)

column chromatography, using antisera raised in rabbits and directed against testosterone-3-(O-carboxymethyl)-oxime- bovine serum albumin. Tritiated [1,2,6,7,16,17-H^3] testosterone (New England Nuclear, Boston, MA, U.S.A.) was used as a radioactive label, while testosterone from Sigma Chemical Company, St. Louis, MO, U.S.A., was used in the range from 0.01 to 15 nmol per tube as the standard preparation. The sensitivity of the assay was 0.1 nmol/l and the precision as calculated from a serum pool (mean value: 1.85 nmol/l) after 14 consecutive measurements, was 3.9% within assay and 5.7% between assay.

Histology

The left testis from each animal was fixed in Bouin's fluid, dehydrated in 70% ethanol and embedded in paraffin wax. Duplicate transverse sections of 5 µm were taken from the mid-portion of the testis and stained with Periodic acid-Schiff reagent with and without prior diastase digestion. For histological measurement we used the germ cell maturation sequence of 14 stages as proposed by Leblond and Clermont (14). The counts were made on cross sections of 25 seminiferous tubules. The numbers of spermatogonia and preleptotene spermatocytes were expressed as numbers per 100 Sertoli cells. Expressing the data this way corrected for tissue shrinkage and permitted a comparison of germ cell counts from one time interval to another. Spermatogonia of types Ao and A1 were scored as a single class of cells since in our material they could not be differentiated. Type B spermatogonia were scored at stage 6; intermediate spermatogonia at stage 3 and preleptotene spermatocytes at stage 8 of the cycle.

Flow-cytometry

The procedure used was similar to that described previously by van Kroonenburgh et al. (10). The right testis from each animal was dissected free from fat and connective tissue. After decapsulation,

the tissue was finely minced with scissors into 15 ml phosphate buffer (pH=7.4; 23.8 mg phosphate/ml) and sieved through a 50 µm mesh filter. The mincing and preparations were carried out at room temperature. This cell suspension was passed through a 25-gauge, 2.5 cm needle, centrifuged (370xg) for 10 min, and resuspended in 10 ml phosphate buffer by vortex mixing. Debris was removed by filtration through a nylon filter (pore size: 50 µm). The suspension was again centrifuged (370xg) for 10 min and the supernatant discarded. All aliquots were fixed in 50% ethanol (-20°C) and stored at 4°C until analysis. To obtain a fluorescent stained single cell suspension, the staining procedure was caried out in the dark at room temperature for 30 min, as previously described by Hamilton et al. (6) Briefly, 1 ml of cell suspension (about $3x10^6$ cells) was centrifuged for 10 min (360xg) and the ethanol discarded. The cell pellet was resuspended in 1 ml staining solution consisting of 0.1 mg mithramycin (MM: Serva Fein Biochemica, Heidelberg, F.R.G.) per ml of 15mmol $MgCl_2$/l in 0.85% NaCl. Flow cytometric analysis was performed on an Ortho System 50-H (Ortho Instruments; Westwood, MA, U.S.A.) using the 457-nm line of the argon ion laser (Spectra Physics, Mountain View, CA, U.S.A.) at 200 mW power. The amount of DNA per cell can be expressed as the c-value, representing the amount of DNA in a gamete.

Statistical analysis was performed by the Kruskal-Wallis one-way analysis of variance to decide whether differences between the groups were statistically significant. The H values obtained were corrected for ties. If the level of significance was <0.05, the Mann-Whitney 'U'-test was performed to make single comparisons with pretreatment values. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

The testis weights after a single s.c. injection of a 500 µg Zoladex depot are shown in Fig. 8.1. No significant decrease in total testicular weight after drug administration was observed.

Zoladex caused a statistically significant decrease in serum FSH, LH and testosterone concentrations, with a maximum effect at day 14 followed by a rapid increase, especially for FSH (130% compared with control value), to a maximum on day 28. Thereafter all values

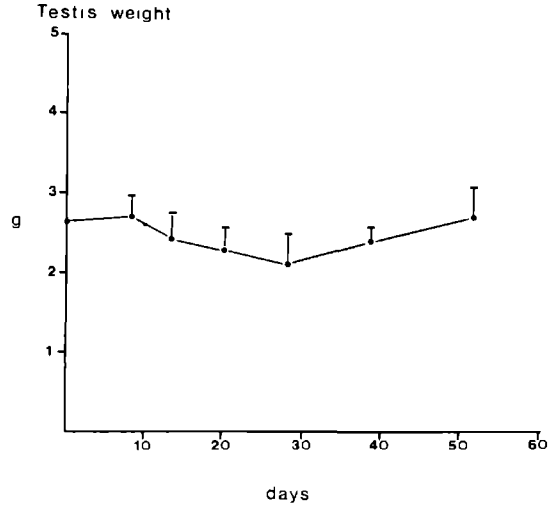


FIG. (8.1) *Effects of a single s.c. depot containing 500 µg Zoladex given at zero time on the weight of testes in adult rats. Values are mean ± s.d. for six rats/group.*

decreased somewhat (Fig. 8.2).

One week after drug administration, degenerative necrotic changes of germ cells as well as Sertoli cells were observed. Some tubules showed disorganization of sperm maturation, disruption of cellular association or total absence of germ cells with preservation of Sertoli cells, whereas other tubules in the same area showed completely normal spermatogenesis (Fig. 8.3).

After 14 days, many tubules showed disruption of cellular association and the tubules were smaller than normal with a greater space between individual tubules, suggesting interstitial oedema. The cellular disruption made quantification using the criteria proposed by Leblond and Clermont (14) impossible between days 7 and

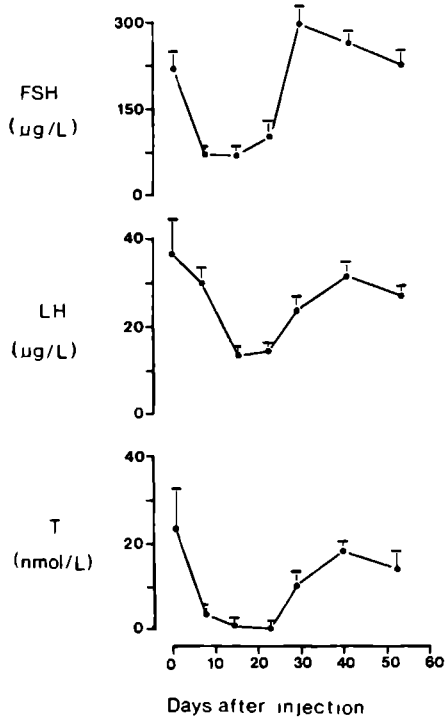


FIG. (8.2) *Effects of a single s.c. depot containing 500 µg Zoladex given at zero time on mean ± s.d. serum concentrations of FSH (upper panel), LH (middle panel) and testosterone (lower panel) in adult male rats (six/ group).*

39. No significant difference was observed between the control numbers of spermatogonia and preleptotene spermatocytes and the numbers of spermatogonia and preleptotene spermatocytes as found on day 52. Mean control numbers (± S.E.M.) of type A, intermediate and type B spermatogonia and of preleptotene spermatocytes per 100 Sertoli cells were respectively 10 ± 1, 58 ± 5, 94 ± 12 and 150 ± 10. Two weeks after Zoladex administration, white plaques of approximately 10 mm diameter were seen in the testes. This was caused by necrotic degenerative changes and/or calcification of seminiferous tubules. In addition many multinucleate giant cells (Fig. 8.3) were seen in the seminiferous tubules. During the whole study there were no obvious morphological changes in Leydig cells.

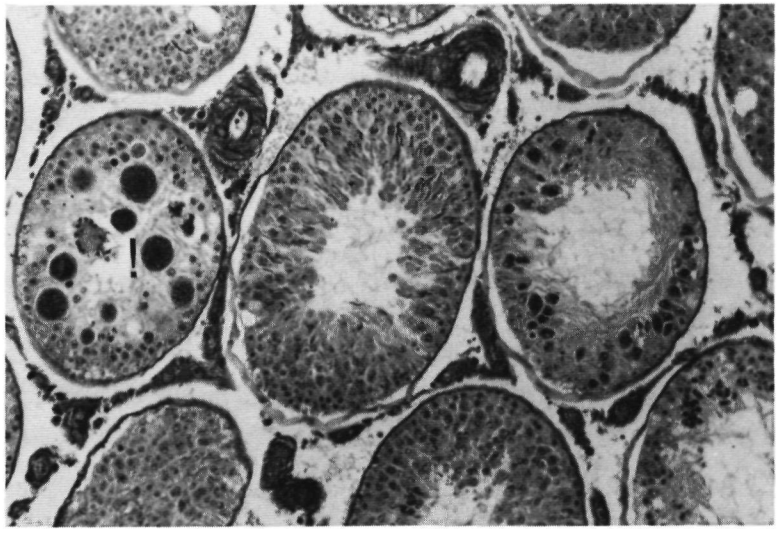


FIG. (8.3) *Testicular morphology of an adult rat, 7 days after administration of a depot containing formulation 500 µg Zoladex (ICI 118630). (Stained with periodic acid-Schiff reagent. Magnification: 135x). Some tubules showed degenerative changes, whereas other tubules showed completely normal spermatogenesis. Note the multinucleated giant cells (!). These cells are associated with degenerative changes of spermatids.*

An example of a flow cytometric histogram of untreated testicular cells is presented in Fig. 8.4.

The two subpeaks in the 1C region of the histogram represent round (1a) and elongated (1b) spermatids respectively (10). The geometry of the histograms was symmetrical and distributions were obtained with a coefficient of variation of 3.5%. The two subpeaks in the 1c region of the histogram represent round (1a) and elongated (1b) spermatids, respectively. The peak in the 2c region represents diploid cells: spermatogonia, preleptotene spermatocytes and non-germ cells such as Sertoli cells, Leydig cells, fibroblasts, endothelial cells and macrophages. The peak in the 4c region (tetraploid cells) consists mainly of pachytene spermatocytes (11).

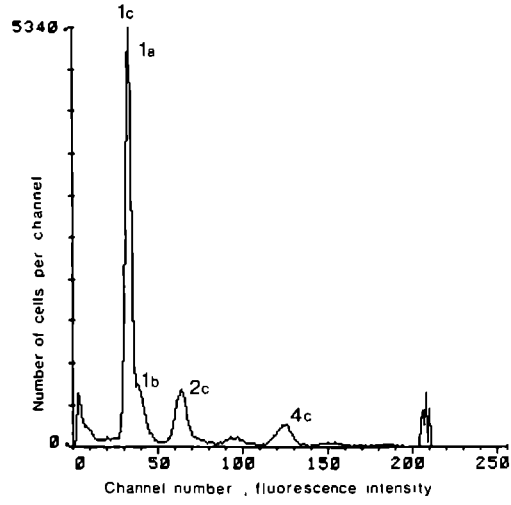


FIG. (8.4) *DNA fluorescence distribution from a single cell suspension of a rat testis. The first peak (1c) represents haploid cells and consist of two subpeaks 1a and 1b which represent round (1a) and elongated spermatids (1b). The second (2c) and third (4c) peaks represent diploid and tetraploid cells, respectively. Cells were stained with mithramycin.*

Fig. 8.5 presents the total number of haploid cells and tetraploid cells and the number of cells in the S-phase. A decrease ($P<0.05$) in total number of haploid cells to 25% of that of controls was observed from day 14 to day 39 after drug administration, almost parallelling a decline in tetraploid cells. A significant ($P<0.05$) decrease in the number of cells in the S-phase to 50% of those of controls was observed only at day 28. Flow cytometric values returned after day 28 to become essentially normal by day 52.

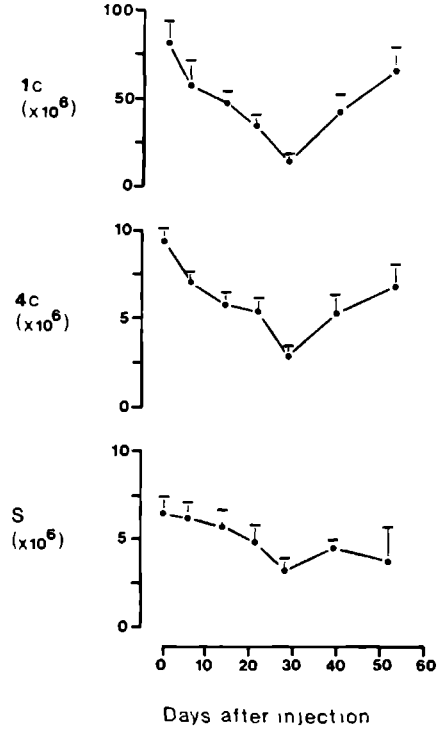


FIG. (8.5) *Mean (± s.d.) absolute numbers of haploid (1c) cells (spermatids; upper panel), tetraploid (4c) cells (mainly pachytene primary spermatocytes; middle panel) and cells in the S-phase of the cell cycle (lower panel) per testis after administration of a single depot containing 500 µg Zoladex. There were six rats per group.*

DISCUSSION

The mechanism responsible for the paradoxical inhibitory effect of LHRH analogues on spermatogenesis appears to be related to their

direct desensitizing effect on the pituitary gland (18). This results in the suppression of LH and FSH release. After administration of the new depot formulation of the LHRH agonist Zoladex, a marked fall in circulating concentrations of gonadotrophins and testosterone was observed. These findings are in contrast with chronic treatment with LHRH analogues in the rat as reported previously. In those studies (5,7) serum concentrations of testosterone were decreased in the presence of normal or even increased serum LH concentrations. These data suggested that the inhibition of testosterone secretion was caused by initial hyperstimulation of LH, which in turn would lead to secondary down-regulation of testicular LH receptors, thereby decreasing testicular steroidogenesis. Our studies indicate a primary effect of chronic analogue treatment at the pituitary level. However, direct effects on steroidogenesis (2,5,13) or on testicular LH and prolactin receptors (1,21) are still possible. The sharp increase in gonadotrophin concentrations at day 28 (Fig. 8.2) with a tendency to overcompensate was probably due to release of pituitary FSH and LH as the pituitary gland is released from the desensitizing effect of the LHRH analogue after the depot became exhausted. In order to achieve complete suppression of gonadotrophins, repeat injections of Zoladex in this dosage at 21 day intervals may be necessary.

The histological and cytological changes observed are interesting. During the period when FSH and LH concentrations were suppressed, focal changes in histology were observed with some tubules being totally degenerative in the neighbourhood of tubules of apparently normal appearance. This phenomenon, which has been described previously (13,19,20), suggests that there may also be a direct effect of the LHRH analogue on the testis (8). However, this degenerative pattern has also been described during spontaneous atrophy and could be related to withdrawal of androgen from the testis (3). It is known that, in addition to their pituitary effects, LHRH analogues also have direct effects on the Leydig cells of the rat (22), although no morphological changes were observed in the Leydig cell in our study. The appearance of multinucleate giant cells (Fig. 8.3) has been documented previously by Burek (3). The origin of these cells is associated with spermatids. Burek (3)

described the formation of multinucleate spermatids as degenerative changes.

Of special interest is the quantitative analysis of changes in proliferative activity of testicular cells. During normal spermatogenesis, the last duplication of DNA takes place in the S-phase of the primary spermatocyte (17). The number of cells in the S-phase therefore reflects the proliferative activity of cells in the early stages of spermatogenesis. From the decrease of cells in the S-phase at day 28 (Fig. 8.5), it can be concluded that the drug influences the proliferative activity of spermatogonia and/or primary spermatocytes, either directly or indirectly. Whether this was a decrease in spermatogonia or in preleptotene spermatocytes, or both, remains to be investigated. Because tetraploid cells in the testis are mainly pachytene spermatocytes, and only to a lesser degree spermatogonia in mitosis (11), Zoladex also seems to influence the number of pachytene spermatocytes (Fig. 8.5).

It can be concluded that Zoladex has a sustained suppressive effect on spermatogenesis in adult rats. This new method of administration may have practical and pharmacokinetic advantages for the purpose of reversible inhibition of spermatogenesis.

ACKNOWLEDGEMENTS

We acknowledge the skilful technical assistance of Mr J. Koedam, Mr H. van Wezel and Dr M.F.G. Segers. We thank Dr S. Raiti of the NIADDK for the gift of the LH and FSH reagents, and Professor F. Debruyne and Dr P. Colla for advice. Dr H. Scholtz is specially acknowledged for his assistance in the histological evaluation of the testicular tissue. Zoladex was kindly supplied by ICI PLC., Pharmaceuticals Division, Macclesfield, Cheshire, UK..

References

1. Auclair C, Kelly PA, Coy DH, Labrie F and Schally AV: Inhibition of testicular luteinizing hormone receptor level by treatment with a potent LHRH agonist or human chorionic gonadotropin. Biochem. Biophys. Res. Commun. 76, 855-862, 1977.

2. Bélanger A, Auclair C, Ferland L and Labrie F: Time-course of the effect of treatment with a potent LHRH agonist on testicular steroidogenesis and gonadotropin receptor levels in the adult rat. J. Ster. Biochem. 13, 191-196, 1980.

3. Burek JD: Pathology of aging rats. Ed. West Palm Beach, FL. U.S.A., CRC Press, Inc. 1978.

4. Corbin A: From contraception to cancer: A review of the therapeutic applications of LH-RH analogues as antitumor agents. The Yale J. Biol. Med. 55, 27-47, 1982.

5. Cusan L, Auclair C, Bélanger A, Ferland L, Kelly PA, Séguin C and Labrie F: Inhibitory effects of long term treatment with a luteinizing hormone-releasing hormone agonist on the pituitary-gonadal axis in male and female rats. Endocrinology. 104, 1369-1376, 1979.

6. Hamilton VT, Habbersett MC and Herman CJ: Microfluorometric analysis of cellular DNA: Critical comparison of mithramycine and propidium iodide. J. Histochem. Cytochem. 28, 1125-1128, 1980.

7. Heber D and Swerdloff RS: Gonadotropin-releasing hormone analog and testosterone synergistically inhibit spermatogenesis. Endocrinology. 108, 2019-2021, 1981.

8. Hsueh AJW and Erickson GF: Extra pituitary inhibition of testicular function by luteinizing hormone releasing hormone Nature 281, 66-67, 1979.

9. Hutchinson FG and Furr BJA: Biodegradable polymers for the sustained release of peptides. Biochem. Soc. Trans. 13, 520-523, 1985.

10. Kroonenburgh MJ, Beck JL, Scholtz JW, Hacker-Klom U and Herman CJ: DNA analysis and sorting of rat testis cells using two-parameter flow cytometry. Cytometry 6, 321-326, 1985.

11. Kroonenburgh MJ, van Gasteren HJ, Beck JL and Herman CJ: A computer model of spermatogenesis in the rat; correlation with flow cytometric data based on autoradiographic cell cycle properties. Cell Tiss. Kinet. 19, 171-177, 1986.

12. Labrie F, Auclair C, Cusan L, Kelly PA, Pelletier G and Ferland L: Inhibitory effects of LHRH and its agonists on testicular gonadotropin receptors and spermatogenesis in the rat. Fifth

annual workshop on the testis: endocrine approach to male contraception. Int. J. Andr. Suppl. 2, 303-318, 1978.

13. Labrie F, Cusan L, Séguin C, Bélanger A, Pelletier G, Reeves J, Kelly PA, Lemary A and Raynaud JP: Antifertility effects of LHRH agonists in the male rat and inhibition of testicular steroidogenesis in man. Int. J. Fert. 25, 157-170, 1980.

14. Leblond CP and Clermont Y: Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55, 548-573, 1952.

15. Linde RB, Doelle GC, Alexander N and Rabin D: Reversible inhibition of testicular steroidogenesis and spermatogenesis by a potent GnRH agonist in normal men: An approach toward the development of a male contraceptive. N. Engl. J. Med. 305, 663-667, 1981.

16. Matsuo H, Baba Y, Nair RMG, Arimura A and Schally AV: Structure of the porcine LH-and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. 43, 1374-1439, 1971.

17. Monesi V: Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis, using tritiated thymidine. J. Cell Biol. 14, 1-18, 1962.

18. Rajfer J, Swerdloff RS and Heber DM: The pituitary gland is the primary site of action of GnRH agonist CD-Leu^{6}, des-Gly^{10}-GnRH ethylamide in the male. Fert. Steril. (Abstract) 38, 277, 1982.

19. Rajfer J, Swerdloff RS and Heber DM: Testicular histology following chronic gonadotropin-releasing hormone agonist treatment. Fert. Steril. 42, 765-771, 1984.

20. Rivier C, Rivier J and Vale W: Chronic effects of [D-Trp^{6}, Pro^{9}, NEt] luteinizing hormone-releasing factor on reproductive processes in the male rat. Endocrinology 105, 1191-1201, 1979.

21. Sharpe RM, Fraser HM and Sandow J: Effect of treatment with an agonist of luteinizing hormone-releasing hormone on early maturational changes in pituitary and testicular function in the rat. J. Endocr. 80, 249-257, 1979.

22. Sharpe RM and Fraser HM: Inhibition of maturational changes in cell function by treatment of rats with an agonist of LH-RH. J. Reprod. Fertil. 60, 359-368, 1980.

23. Van Geelen JM, Doesburg WH, Thomas CMG and Martin CB: Urodynamic studies in the normal menstrual cycle: The relationship between hormonal changes during the menstrual cycle and the urethral pressure profile. Am. J. Obstet. Gyn. 141, 384-392, 1981.

CHAPTER 9

SURVIVAL OF SPERMATOGONIAL STEM CELLS IN THE RAT AFTER SPLIT DOSE IRRADIATION DURING LH-RH ANALOGUE TREATMENT

M.J.P.G. van Kroonenburgh, W.A.J. van Daal, J.L. Beck, H.M. Vemer, R. Rolland and C.J. Herman.

(Submitted for publication in Journal of the European Society for Therapeutic Radiology and Oncology)

ABSTRACT. A rat model has been created in which a single injection of an LH-RH analogue depot preparation (Zoladex, ICI 118630) produced a temporary interruption of the pituitary-gonadal axis. This effect applied during irradiation was investigated as a possible mechanism to protect the testis from radiation damage. A local testicular irradiation dose of 6.0 Gy was given either as a single dose or as a fractionated (2 x 3.0 Gy) dose at different time intervals ranging from 8 to 72 hours. Stem cell survival was measured 11 weeks after irradiation by means of the repopulation index and the number of haploid cells (spermatids) measured by flow cytometry. Serum gonadotrophins and testosterone concentrations were measured to evaluate hormonal suppression. No significant differences were observed between serum concentrations of FSH, LH and testosterone and the duration of the fractionation interval. Stem cell survival was higher following fractionated irradiation in comparison with the single dose. For the 8 hr interval an increase in recovery ratio was found, amounting to 5.0 of the single dose value. The fluctuating pattern of the recovery curves indicated changes in radiosensitivity of stem cells. The combination of hormonal inhibition of spermatogenesis and fractionated irradiation led to a decrease in the absolute numbers of stem cells. However, the stem cell recovery curves were identical to those seen without hormonal inhibition. It was concluded that hormonal pretreatment with Zoladex during split dose irradiation had no protective effect on stem cell survival.

INTRODUCTION

Although the spermatogonial stem cells appear to be radioresistant cells (3), their state of radioresistance is probably not a permanent one (6,10,18,33,34,37). In CBA the mouse 16 hours after a single dose of irradiation stem cells showed increased radiosensitivity, which reaches a maximum at 20 hours (34). Fractionated irradiation could in this way lead to stem cell depletion (33,35) by killing stem cells in their radiosensitive phase. A survey of the literature on the survival of testicular stem cells after fractionated irradiation, however, showed conflicting results. Withers et al. (37) and Lu et al. (23) described enhanced stem cell survival when the dose was split into two equal fractions, whereas Oakberg (21) and Cattanach (2) reported no consistent changes. The conflicting data on stem cell survival after split dose irradiation may be attributed to the use of different mouse strains (27) or to the different methods (23) used in measuring stem cell survival. Spermatogonial stem cell survival after irradiation was evaluated in this study by the repopulation index. This method was used previously (18,23,32,37) and a good correlation was shown (32) between this index and stem cell survival. Sonication-resistant spermhead counts may also provide a reasonable measure of stem cell survival (25,26). Because the number of haploid cells corresponds to the number of spermatids, the number of haploid cells as measured by flow cytometry can also be used as a method to measure stem cell survival (14).

In the present study we attempted to protect testicular stem cells during irradiation by prior administration of the LH-RH analogue depot preparation Zoladex. Temporary interruption of the pituitary-gonadal axis by a hormonal drug may prevent stem cells from moving into their radiosensitive phase after irradiation. Inhibition of follicle stimulating hormone (FSH) and/or luteinizing hormone (LH) release or inhibition of their effects on the testicle could place the testis in a resting state and could make the stem cells more resistant to irradation. This could protect stem cells from depletion resulting in preservation of reproductive function. Data from the literature in preserving testicular function by

hormones during irradiation are very scarce. A recent study (16) showed, however, protective effects on the ovaries of rats, when an LH-RH analogue was given during irradiation. Concerning the protective effect of hormonal inhibition on testicular damage due to chemotherapy more data are available (8,11,12,17,22,29).

MATERIALS AND METHODS

Zoladex 118630 (ICI PLC., Pharmaceuticals Division, Macclesfield, Cheshire, U.K., a depot LH-RH analogue, is a potent pituitary gonadotrophin inhibitory agent (7,21) and we therefore used this compound to interrupt the pituitary-gonadal axis during irradiation. In a previous study (20) a single injection of Zoladex produced a marked decrease in serum concentrations of FSH, LH and testosterone with a maximum effect 14 days after treatment. Testis weight, serum hormone concentrations, and histological, and cytological parameters were returned to essentially normal values 52 days after the injection. Three-month-old male outbred Wistar rats (Cpb:WU) each weighing about 300 g were housed in groups of 4 in well ventilated cages at 22°C with a day/night rhythm of 12 hours. The animals were allowed free access to dry pellet food (RMH-TM HOPE-FARMS, Woerden, The Netherlands) and tap water. 108 rats were divided into two groups: Group 1 (54 rats), received a single injection of 500 µg Zoladex 14 days prior to the start of testicular irradiation procedures. Group 2 (54 rats), were irradiated without any pretreatment. Testicular irradiation was given either as a single dose of 6.0 Gy or split into two doses of 3.0 Gy with time intervals of respectively 8, 16, 20, 24, 28, 36, 48 or 72 hours. The rats were killed at 10.00h under ether anaesthesia 11 weeks after the irradiation procedure. Blood was collected by heart puncture and the testes were removed immediately and weighed. After centrifugation (1200g, 10 min) serum samples were stored at -20°C until analysed.

Irradiation conditions

The rats were anaesthesized with an Alyrane (2-chloor-1,1,2-trifluorethyl-difluor methylether) oxygen gas mixture and irradiated in prone position with the 18 MV photon beam of a Saturne linear accelerator (CGR, BUC, France). The Saturne is equipped with a mercury shielded irregular field system device (4) which was used to define 4 irradiation portals of 4x5 cm each, so that 4 rats could be irradiated on their scrotum simultaneously. To achieve a dose homogeneity in the target volume within 10% it was necessary to get rid of the build region in the beam. Therefore a 1.5 cm thick plexiglas plate was positioned just above the rats. The dose rate during the experiments was 0.6 Gy/minute.

Stem cell survival assays

Spermatogonial stem cell survival after irradiation was evaluated by the repopulation index and by counting the number of haploid cells (spermatids) by using flow cytometry. The left testis from each sacrificed animal was fixed in Bouin's fluid and embedded in paraffin. From each testis three sections, separated from each other by a distance of 500 µm, were taken from the midportion of the testis and stained with Periodic Acid-Schiff (PAS). The percentage of tubules which showed spermatogenic repopulation in histological sections, 11 weeks after irradiation, was defined as the the repopulation index (23). A tubule was considered to repopulate when it contained at least one spermatogonium. The right testis from each animal was dissected free from fat and connective tissue. To obtain a fluorescent stained single cell suspension, the preparation and staining procedure was carried out as previously described (12). Haploid cells were measured by flow-cytometric analysis on an Ortho System 50-H (Ortho Instruments; Westwood, MA, U.S.A) using the 457 nm line of the argon ion laser (Spectra Physics, Mountain View, CA, U.S.A) at 200 mW power. The stem cell recovery ratio was calculated as the ratio of the repopulation index or the absolute number of haploid cells in the fractionated and unfractionated irradiated

testes.

Hormone measurements

Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double-antibody solid phase radioimmunoassays using the rat gonadotrophin kit reagents supplied by NIAMDD, Bethesda, MD, U.S.A. Measurements were expressed as µg/l in terms of the NIADDK-rat FSH-RP-1 and LH-RP-1 standards, respectively. The minimum detectable concentrations were 10 µg LH/l and 50 µg FSH/l. The precision was 5.9% within assays and 6.7% between assays for duplicate measurements of FSH in a serum pool (mean value 264 µg/l) in 15 consecutive assays. For LH these values were, respectively, 3.9% and 9.6% (mean: 59 µg/l) in 15 consecutive assays. Testosterone was measured by a dextran-coated charcoal (DCC) radioimmunoassay after extraction of serum samples with diethyl ether. The antiserum used was raised in a rabbit and directed against Testosterone-3-(O-carboxymethyl)-oxime-BSA. The sensitivity of the assay was 0.1 nmol/l and the precision, as calculated from a serum pool (mean value: 1.85 nmol/l) after 14 consecutive measurements), was 3.9% within assays and 5.7% between assays.

Statistical analysis was performed by using the two-sided Mann-Whitney U test. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

Testicular weights did not show any statistically significant differences between two equal doses (3.0 Gy) of irradiation, separated by different time intervals ranging from 0 to 72 hours (Fig. 9.1).

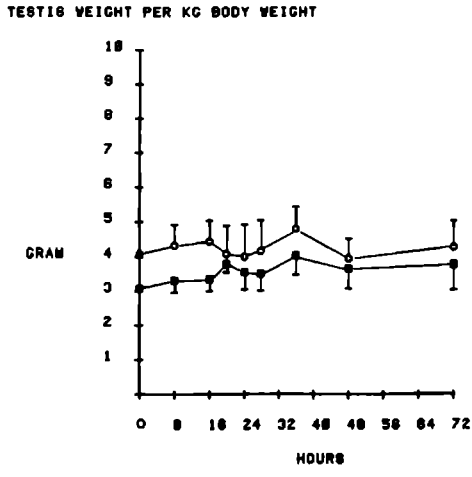


FIG. (9.1) *Effects of split dose irradiation (2 x 3.0 Gy) at different time intervals with (■) or without (o) Zoladex depot pretreatment on mean testicular weight of adult Wistar rats (mean ± SD; 6 rats/group) 11 weeks after radiation in this and the following figures.*

Although the mean testis weight in the rats pretreated with Zoladex tended to be lower than in the untreated rats, the difference was not significant. The spermatogenic stem cell survival as measured by the repopulation index and the number of surviving haploid cells (spermatids) 11 weeks after split dose irradiation (2 x 3.0 Gy) at various time intervals are presented in Fig. 9.2.

The two stem cell survival assays showed good agreement and the curves showed a fluctuating pattern with a significant ($P < 0.05$) dip in the recovery ratio at 20 hours. A second dip could be observed 20 hours later. Recovery ratio of stem cells after split dose of irradiation (2 x 3.0 Gy) during interruption of the pituitary-gonadal axis by Zoladex is presented in Fig. 9.3.

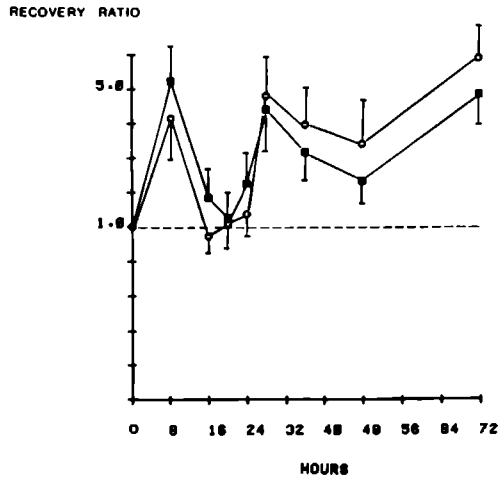


FIG. (9.2) *Stem cell survival as measured by the number of haploid cells (spermatids, o) and the repopulation index (■) after split dose irradiation at different time intervals in adult rats (mean ± SD; 6 rats/group).*

The stem cell recovery curves showed a fluctuating pattern, nearly identical to the curves without Zoladex. However, the absolute number of haploid cells and repopulation index were significant ($P< 0.05$) different. At the irradiation dose of 6.0 Gy (time interval 0 hour) $2.0x10^6 \pm 1.6x10^5$ haploid cells were measured 11 weeks after irradiation, whereas the number of haploid cells was $4.0x10^5 \pm 2.4x10^4$ after hormonal pretreatment, suggesting a 5 fold decrease in the number of stem cells. At the irradiation dose of 6.0 Gy (time interval 0 hour) the repopulation index in the group of rats with Zoladex pretreatment significant ($P< 0.05$) decreased from 13.3±1.2% to 4.3±0.6%. The serum hormone concentrations of FSH, LH and testosterone after 11 weeks are shown in Fig. 9.4. No significant differences were observed between serum concentrations of FSH, LH and testosterone and the duration of the fractionation interval. Comparison with previous data (20) from control rats

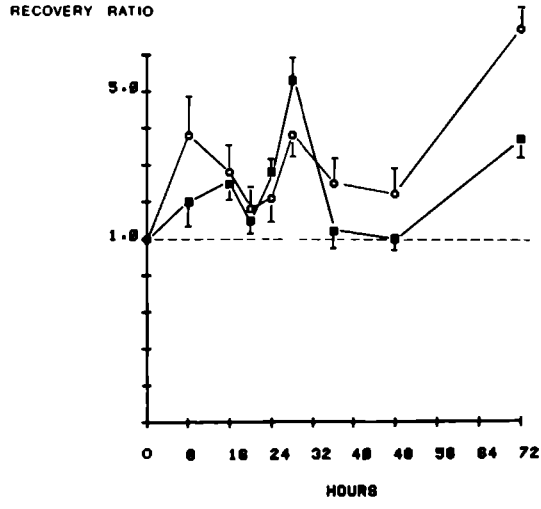


FIG. (9.3) *Stem cell survival as measured by the number of haploid cells (spermatids, o) and repopulation index (■) after split dose irradiation with different time intervals in adult rats (mean ± SD) pretreated at 14 days prior to irradiation with a single zoladex depot (500 µg) injection 6 rats/group.*

showed, that the found serum concentrations of FSH, LH and testosterone were not statistically different ($P < 0.05$) from control values.

DISCUSSION

Our results show enhanced stem cell survival after fractionated radiation at time intervals less than 72 hours as measured by two different stem cell assays. These results are in accordance with the results of Withers et al. (37) and Lu et al. (23). In these studies, however, mice were used. The fluctuating pattern of the stem cell recovery curves (see Figs. 9.2 and 9.3) may be related to fluctuations in radiosensitivity of these cells. This fluctuation in

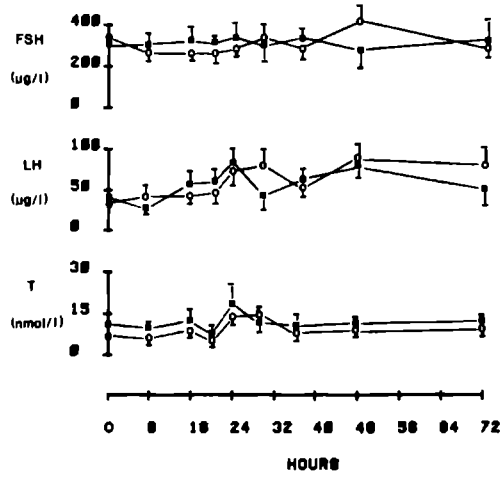


FIG. (9.4) *Effects of split dose irradiation (2 x 3.0 Gy) with (o) or without (■) Zoladex depot (500 µg) pretreatment in Wistar rats on mean serum concentrations of FSH (upper panel), LH (middle panel) and testosterone (lower panel), 11 weeks after irradiation.*

radiosensitivity could be attributed to a move of stem cells toward a more radiosensitive phase of their cell cycle (6,10,33,34,37). In this way, the dip in the curve at 20 hours may reflect the moment at which the stem cell enters the very radiosensitive (10) mitotic phase. The second dip may then suggest the mitotic phase of the next stem cell cycle. The testicular weight showed no significant differences between time intervals, indicating that this parameter is of little value in estimating stem cell survival. Of special interest was the combination of fractionated irradiation with hormonal suppression by Zoladex. The split-dose recovery curve showed a similar fluctuating pattern. Zoladex had apparently no influence on the moment of occurrence of the dip. It seemed that the remaining stem cells behaved as if no hormonal suppression had been given, suggesting that the stem cells are not under endocrine control or the degree of suppression is not great enough to inhibit

proliferation. However, during hormonal suppression a distinct decrease in the number of stem cells occurred. This decrease needs further exploaration. After the single dose of 6.0 Gy (time interval 0 hours) the number of stem cells was also decreased, from which we conclude that the decrease in the number of stem cells probably has little or nothing to do with a move toward a different phase in their cell cycle. Although the Zoladex pretreatment combined with radiation did reduce the haploid count by a factor 5, it is difficult to say if this is a direct five-fold decrease in stem cell number. The relationship between spermatid production and stem cell number may be linear, however, for the mouse (26), it has been demonstrated that although sperm production and stem cell survival as measured by RI were highly correlated the relationship was not linear. By measuring the number of haploid cells (spermatids) as an endpoint for stem cell survival, we disregard the fact that all other spermatogenic cells between stem cells and spermatids may influence this parameter. In other words, intervention in the process of spermatogenesis, could make the number of haploid cells inadequate as a stem cell survival assay. This is, however, not the case with the repopulation index and since the repopulation index also decreased it is very likely that the observed decrease in the number of stem cells is real.

Gonadal dysfunction is an important side effect of radiation therapy (13,24). In male patients it can result in permanent azoospermia or severe oligospermia (1,2,15,28,31). Even when the testes are shielded, internal scatter is still a significant problem (36). A treatment which reduces the magnitude of testicular damage after irradiation is highly desirable and could result in preservation of reproductive function. The agonist LH-RH ethylamide has been shown (11,12) to protect the murine testis from damage by exposure to cyclophosphamide. The study of Johnson et al. (17), however, showed no improvement in post treatment fertility in men after chemotherapy when they were treated with an LH-RH analogue. The present results lead to the conclusion that the prior administration of an LH-RH analogue like Zoladex does not produce any substantial improvement in recovery of fertility after irradiation. We can not exclude, however, that there may be a

protective effect at higher irradiation dose or when the dose is multifractionated. Our results indicated that a fractionation interval of 8 hours could result in reducing gonadal damage in comparison with the 20 hour interval. Of interest in this respect is the study of Deringer et al. (9), who observed already in 1954 a regain of fertility in mice when time intervals between fractions of irradiations of 8 hours were used. Clinical radiation therapy, for instance in Hodgkin's disease, frequently employs time intervals of about 24 hours, which could destroy stem cells in the most radiosensitive phase. Extrapolation of our results to humans or even to other species is difficult and further studies are necessary to explore this in more detail.

In the present study we conclude that in Wistar rats changes in stem cell radiosensitivity occur most likely in relation to cell cycle alterations in the radiation response of the stem cells. Stem cell survival was enhanced after split doses of irradiation compared with the same dose unfractionated. Hormonal pretreatment with an LH-RH analogue depot preparation Zoladex during split dose irradiation had no protective effect on stem cell survival.

ACKNOWLEDGEMENTS

We acknowledge the skilful technical assistance of Mr J. Koedam and Mr H. van Wezel; the advice of Dr H.J. van Gasteren; the gift of the LH and FSH reagents by Dr S. Raiti of NIAMDD, Bethesda, MD, U.S.A. and we thank Dr C.M.G. Thomas and Dr M.F.G. Segers Dept. of Obstetrics and Gynaecology, Nijmegen, for measuring FSH, LH and testosterone concentrations in serum. Zoladex was kindly supplied by ICI. PLC., Pharmaceuticals Division, Macclesfield, Cheshire, U.K..

References

1. Ash P: The influence of radiation on fertility in man. Br. J. Radiol. 53, 271-278, 1980.

2. Berthelsen JG: Sperm counts and serum follicle-stimulating hormone levels before and after radiotherapy and chemotherapy in men with testicular germ cell cancer. Fertil. and Steril. 41, 281-286, 1984.

3. Bianchi M: Cytotoxic insult to germinal tissue. In: Potten CS, Hendry JH (eds). Cytotoxic insult to tissue. Churchill Livingstone, Edinburgh, 1983.

4. Brouwer WFM, Kazem I and Mil van CJHM: Four years experience with a mercury shielded irregular field system (MSIFS). Int. J. Radiat. Oncol. Biol. Phys. 10, 225-229, 1984.

5. Cattanach BM: Spermatogonial stem cell killing in the mouse following single and fractionated X-ray doses, as assessed by length of sterile period. Mut. Res. 25, 53-62, 1974.

6. Clow DJ and Gillette EL: Survival of type A spermatogonia following X-irradiation. Radiat. Res. 42, 397-404, 1970.

7. Cusan L, Auclair C, Bélanger A, Ferland L, Kelly PA, Séguin C and Labrie F: Inhibitory effects of long term treatment with a luiteinizing hormone-releasing hormone agonist on the pituitary-gonadal axis in male and female rats. Endocrinology. 104, 1369-1376, 1979.

8. Delic JI, Bush C and Peckham MJ: Protection from procarbazine-induced damage of spermatogenesis in the rat by androgen. Canc. Res. 46, 1909-1914, 1986.

9. Deringer MK, Heston WE and Lorenz E: Effects of long-continued total body y-irradiation on mice, guinea pigs and rabbits, IV. Actions on the breeding behavior of mice in, R.E. Zirkle (ed.), Biological Effects of External X-and γ-Radiation, Mc Graw-Hill, New York, 149-168, 1954.

10. Erickson BH: Effect of Co^{60}-γ-radiation in the stem and differentiating spermatogonia of the post pubertal rat. Radiat. Res. 68, 433-448, 1976.

11. Glode LM, Robinson J, Gould SF, Nett TM and Merril D: Protection of spermatogenesis during chemotherapy. Drugs Exptl. Clin. Res. 7, 367-378, 1980.

12. Glode LM, Robinson J, Gould SF: Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropin-releasing hormone. Lancet. I, 1132-1134, 1981.

13. Greiner R: Die Erholung der Spermatogenese nach fraktionierter, niedrig dosierter Bestrahlung der mannlichen Gonaden. Strahlentherapie 158: 342-355, 1982.

14. Hacker-Klom U: Long term effects of ionizing radiation on mouse spermatogensis. Acta Radiol. Oncol. 24, 363-367, 1985.

15. Hahn EW, Feingold SM, Simpson L and Batata M: Recovery from aspermia induced by low-dose radiation in seminoma patients. Cancer 50, 337-340, 1982

16. Jarell J, Younglai EV, Barr R, O'Connel G, Belbeck L and McMahon A: The protection of ovarian follicles from ionizing radiation with agonist LHRH ethylamide. (Abstract) Presented at the 67 th Annual Meeting of the Endocrine Society, Baltimore, Maryland, U S.A., 1985.

17 Johnson DH, Linde R, Hainsworth JD, Vale W, Rivier J, Stein R, Flexner J, van Welch R and Greco A. Effect of a luteinizing hormone releasing hormone agonist given during combination chemotherapy on posttherapy fertility in male patients with lymphoma: Preliminary observations. Blood. 65, 832-836, 1985.

18. Kramer MF, Davids JAG and van der Ven TPA. Effect of 1 MeV fast-neutron irradiation on spermatogonial proliferation in mice Influence of dose fractionation with different intervals. Int. J. Radiat Biol. 25, 253-260, 1974.

19. Kroonenburgh van MJ, Beck JL, Scholtz, JW, Hacker-Klom U and Herman CJ. DNA analysis and sorting of rat testis cells using two-parameter flow cytometry Cytometry 6, 321-326, 1985.

20. Kroonenburgh van MJ, Beck J, Vemer HM, Thomas CMG, Rolland R and Herman CJ: Effects of a single injection of a new depot formulation of an LH-releasing hormone agonist on spermatogenesis in adult rats. J. Endocr., In press, 1986.

21. Labrie F, Cusan L, Séguin C, Belanger A, Pelletier G, Reeves J, Kelly PA, Lemary A and Raynaud JP: Antifertility effects of LH-RH agonists in the male rat and inhibition of testicular steroidogenesis in man. Int. J. Fertil. 25, 157-170, 1980.

22. Lewis RW, Dowling KJ and Schally AV D-Tryptophan-6 analog of luteinizing hormone-releasing hormone as a protective agent against testicular damage caused by cyclophosphamide in baboon. Proc Natl Acad Sci. USA 82, 2975-2979, 1985.

23. Lu CL, Meistrich ML and Howard DT: Survival of mouse testicular stem cells after γ or neutron irradiation. Radiat Res. 81, 402-415, 1980.

24. Lushbaugh CC and Casarett GW: The effects of gonadal irradiation in clinical radiation therapy: A review. Cancer 37, 1111-1120, 1976.

25. Meistrich ML, Hunter, NR, Suzuki N, Trostle, PK and Withers HR: Gradual regeneration of mouse testicular stem cells after exposure to ionizing radiation. Radiat. Res. 74, 349-362, 1978.

26. Meistrich ML: Quantitative correlation between testicular stem cell survival, sperm production, and fertility in the mouse after treatment with different cytotoxic agents. J. Androl. 3,

58-68, 1982.

27. Meistrich ML, Finch M, Lu CC, de Ruiter-Bootsma AL and de Rooij DG: Strain differences in the response of mouse testicular stem cells to fractionated radiation. Radiat. Res. 97, 478-487, 1984.

28. Nader S, Schultz PN, Cundiff JH, Hussey DH and Samaan NA: Endocrine profiles of patents with testicular tumors treated with radiotherapy. Int. J. Rad. Onc. Biol. Phys. 9, 1723-1726, 1983.

29. Nseyo HO, Huben RP, Klioze SS and Pontes JE: Protection of germinal epithelium with luteinizing hormone-releasing hormone analogue. J. Urol. 134, 187-190, 1985.

30. Oakberg EF: Differential spermatogonial stem cell survival and mutation frequency. Mut. Res. 50, 327-340, 1978.

31. Pedrick J and Hoppe RT: Recovery of spermatogenesis following pelvic irradiation for Hodgkin's disease. Int. J. Rad. Onc. Biol. Phys. 12, 117-121, 1986.

32. Ruiter-Bootsma de AL, Kramer MF and de Rooij DG: Response of stem cells in the mouse testis to fission neutrons of 1 MeV mean energy and 300 KV X-rays. Methodology, dose response studies, relative biological effectiveness. Radiat. Res. 67, 56-68, 1976.

33. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1 MeV mean energy of 300 KV X-rays. Radiat. Res. 71, 579-592, 1977.

34. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1 MeV mean energy. Effect of the fractionation interval. Radiat. Res. 79, 289-297, 1979.

35. Sheridan W: The effects of the time interval in fractionated x-ray treatment of mouse spermatogonia. Mut. Res. 13, 163-169, 1971.

36. Speiser B, Rubin P and Casarett G: Aspermia following lower truncal irradiation in Hodgkin's disease. Cancer 32, 692-698, 1973.

37. Withers HR, Hunter NR, Barkley HT and Reid BO: Radiation survival and regeneration characteristics of spermatogenic stem cells of the mouse testes. Radiat. Res. 57: 88-103, 1974.

CHAPTER 10

DISCUSSION AND CONCLUSIONS

Infertility represents one of the main side effects of both chemotherapy and irradiation in cancer (42). The threat of reduced fertility after radiation exposure has assumed increasing importance in view of the increasingly succesful experience in achieving longer survival in cancer radiotherapy patients. A treatment which would reduce testicular damage and hasten the recovery of reproductive function would enhance our ability to bring the treated cancer patient back to a normal life (14).

The aim of this thesis was to test the hypothesis that temporary interruption of the pituitary-gonadal axis by hormone like substances might protect spermatogonial stem cells during irradiation. This hypothesis was supported by experiments which showed, either by counting the spermatogenic repopulation in histological sections of the testes (39,58,59) or by the delayed return of fertility (61), that spermatogonial stem cells became more radiosensitive after a first dose of irradiation. Surviving stem cells are stimulated to divide after irradation (30) and may than be at increased risk. Fractionated irradiation could lead in this way to less stem cell survival by killing stem cells in their radiosensitive phase. Hence the testis may be caught in a destructive cycle and stem cell depletion will occur (58,61), which eventually results in loss of fertility. Reducing the rate of spermatogenesis by hormones could bring the testis into a resting state comparable to prepuberty, which may prevent stem cells from moving into the radiosensitive phase. This might protect stem cells from depletion and hasten the recovery of the reproductive function. Matus et al. (43) showed that prepubertal males were indeed less vulnerable to gonadal damage due to irradiation or chemotherapy in comparison to adults. When discussing testicular irradiation effects particular emphasis should be placed on spermatogonial proliferation and on the importance of the stem cell (52,53). The irradiation of the stem cell is primarily responsible for the

effects of radiation on fertility. This is due to the extreme radiosensitivity of spermatogonia. After a moderate dose of irradiation most spermatogonia are killed. As a result a characteristic maturation depletion can be observed. The testis becomes selectively depleted first from spermatogonia and later from spermatocytes and spermatids. The only cell which is capable of producing new spermatids after irradiation is the relatively radiation resistant stem cell.

Data from the literature in preserving testicular function by hormones during irradiation are very scarce. A recent study (33) showed, however, protective effects on the ovaries of rats, when an LH-RH analogue was given during irradiation. Concerning the protective effect of hormonal inhibition on testicular damage due to chemotherapy more data are available. The agonist LH-RH ethylamide has been shown (14,15) to protect the murine testis from damage by exposure to cyclophosphamide. The study of Johnson et al. (35), however, showed no improvement in post treatment fertility in men after chemotherapy when they were treated with an LH-RH analogue.

Studies to investigate spermatogonial proliferation kinetics have primarily been performed by microscopical examination of testicular tissue (6,7,11,12,29,31,36,37,50), by autoradiography (2,19,20,21,22,24,25,26,27,28,49,51,52) and more recently by flow cytometry (3,4,16,17,40,45,46,54). In the present study we investigated spermatogonial proliferation by microscopical examination and by means of flow cytometric analysis of testis cell suspensions. To estimate stem cell survival different assays can be used as summarized by Lu et al. (41). The various assays have certain advantages as well as disadvantages. For instance counting stem cells does not indicate which stem cell will be functional. Lu et al. (41) suggested that the conflicting results of stem cell survival after split dose irradiation could be attributed to the use of different stem cell assays. To rule out this problem in the present study we therefore used two different stem cell assays: repopulation index and the number of haploid cells.

With the aid of a computer model of spermatogenesis (Chapter 4) we tried to find out to what extent the various stages of the rat

spermatogenic cycle could be characterized by their fluorescence distribution as measured by flow cytometry. The results from this simulation model clearly demonstrated that the processes responsible for the changes in fluorescence intensities were primarily a reflection of germ cells in the spermatocyte and spermatid stages of development and of non-germ cells. Despite the high proliferation rate of spermatogonia their numbers contributing to the relative proportions of cells within the diploid and tetraploid region were very low: 1.25% and 2.0%, respectively. The percentage of the number of spermatogonia contributing to the relative proportions of cells within the S-phase was calculated by the computer model as 43.0%. Thus the only single parameter in DNA measurement which might be sensitive enough to reflect early or small changes in the stem cell and spermatogonial compartment appeared to be the S-phase. However, it remained hard to say if changes in the percentage of S-phase were attributable to the number of spermatocytes or to that of spermatogonia or both.

Another feature which was derived from the computer model was the finding that one stem cell was able to produce 3185 spermatids. This offered the possibility to calculate the total number of stem cells per testis in the rat. In a normal non-treated testis about 70 - 90 million spermatids were found. This resulted in a number of about 21,000 - 28,000 stem cells per testis. This number has also been estimated in the mouse by Meistrich et al. (48). By counting stem cells in a whole tubule, these authors estimated a number of about 20,000 stem cells. So the total number of stem cells is apparantly of the same order in these two species. The time required for the development of stem cells to the beginning of spermatids was calculated by the model to be 40 days. During this development a high percentage of cells died, especially during spermatogonial proliferation. By changing these degeneration percentages in the computer model very little effect in the total percentages of diploid, S-phase or tetraploid cells was observed, probably due to the very low numbers of spermatogonia in comparison with other spermatogenic cells. From the model it also became evident that the detection or quantitation of spermatogonial stem cells with flow cytometric analysis of testicular cell suspensions is impossible.

These calculations were based on flow cytometric analysis using the single parameter of total DNA stainability. Differences in chromatin structure as well as the size of the nucleus of the various germ cells could also be measured and analyzed as separate parameters.

Using two-parameter flow cytometry (chapter 5) of rat testis cell suspensions more subpopulations could be distinguished. This gave more detailed information as compared to single parameter DNA flow cytometry. In this way eight subpopulations were detected and analyzed. This distinction allowed separate analysis of effects on germ cells. Using this two-parameter DNA analysis it was also possible to distinguish non-germ cells from germ cells. Concerning spermatogonial proliferation and stem cell proliferation this distinction gave us no extra information, however, of interest were the binucleated cells with typical bean-shaped nuclei and a very dense nuclear chromatin structure. These cells have an octaploid DNA content. It is not likely that the relatively large sized octaploid cells were stem cells, although this is not totally impossible. These cells could be compared with the giant stem cells as described by Erickson (12). To prove this further research is needed.

The relationship between the rate of recovery of sperm production after irradiation and stem cell survival (47) makes it possible to use flow cytometric measurements as a reliable and rapid method for an indirect quantitative estimation of stem cell survival by counting the number of haploid cells. Haploid cells correspond to spermatids and to no other testicular cells. Theoretically the total number of tetraploid cells can also be used for this purpose. According to the computer model tetraploid cells consist for 98% of pachytene spermatocytes. However, this may not be the case after irradiation of the testis. The relative proportion contributing to the tetraploid region (spermatogonia and pachytene spermatocytes) could change by irradiation making this method inadequate for quantitative estimation of stem cell survival.

Using the number of haploid cells as a stem cell assay, a stem cell survival curve was obtained (chapter 6). A mean Do value for the stem cell in the Wistar rat was calculated as 2.08 Gy. This

value was compared with another stem cell assay (repopulation index), which has been shown previously (57) to correlate very well with stem cell survival. The Do value found with this assay was 2.33 Gy. These results were different from the data of Clow and Gilette (7) and Erickson (12) who found higher Do values for rats (3.8 Gy and 3.7 Gy, respectively). Our data in the Wistar rat are in agreement with those of Meistrich et al.(48), Ruiter-Bootsma et al. (58) and Withers et al. (64) who found Do's of 1.96, 2.42 and 1.80 Gy, respectively. The low Do value found in the Wistar rat could possibly be explained by the differences between the strains. In our studies we used Wistar rats, whereas Clow and Gilette (7) and Erickson (12) used Spraque-Dawley rats.

The effects of a single dose of 3.0 Gy X-irradation on the testis of the rat showed that minimal numbers of spermatogonia were observed within the first two weeks after irradiation. A maturation depletion occurred, which resulted in loss of sperm. It was noticed that recovery of spermatids started 52 days after the irradiation. Theoretically, if all the surviving "As" spermatogonia started differentiating immediately, it would require 40 days to produce sperm (computer model, chapter 4), so a time delay of more than 12 days occurred before the initiation of repopulation. According to the stem cell renewal model of Huckins (28), there are two types of stem cells: one with a short cycle (about 40 hours) and the other, probably the true stem cells, with a much longer cell cycle (about 13 days). Our findings suggested that a single dose of irradiation of 3.0 Gy killed the stem cell with the short cycle. The time delay of more than 12 days could be explained either by the fact that only stem cells with the long cycle (13 days) survived this dose or that stem cells first repopulate themselves within 12 days rather than starting differentiating immediately. It has been shown, however, by Withers et al. (64) that repopulation of stem cells in mice did not occur within several weeks after irradiation. If all stem cells survived and no enhancement occurred, theoretically spermatogenesis would be fully recovered after irradiation in 65 days (Chapter 4). Of interest was the number of spermatids at day 65 after this 3.0 Gy irradiation dose. This number was 62% of the normal value, suggesting that at least 62% of the stem cells escaped the radiation

effect.

As far as the hormonal environment following irradiation is concerned, several studies reported an increase of FSH (1,9,23,34,63), depending on the used dose. Conflicting results, however, have been reported when the dose was about 3.0 Gy. (8,23,34,63). Our hormone measurements confirmed the observations of Cunningham and Huckins (8) and Verjans and Eik-Nes (63) that at this dose the absence of spermatogonia, spermatocytes or spermatids did not affect serum hormone concentrations of FSH and LH.

To interrupt the pituitary-gonadal axis two hormone-like substances were tested: The first drug, Danazol has been shown (chapter 7) to suppress effectively the pituitary-gonadal axis. This was obtained by daily subcutaneous injections of 4 mg Danazol. At this dose a marked and rapid drop in serum testosterone was seen to very low levels with a slower decrease in serum FSH and LH and testis weight. We showed the inhibition of spermatogenesis primarily to take place after the preleptotene stage of primary spermatocytes. The decrease in the number of cells in the S-phase suggested that Danazol inhibits also proliferation of spermatogonia and/or primary spermatocytes.

The second drug, a new depot LH-RH analogue (Zoladex) has also been shown (chapter 8) to have a suppressing effect on the pituitary-gonadal axis. This depot formulation was given as a single subcutaneous injection (500 µg). After injection, the drug slowly degrades and the LH-RH analogue is released into the systemic circulation, resulting in a marked decrease in serum concentrations of FSH, LH and testosterone. The suppression with a maximum effect at 14 days after drug injection was reversible. Testis weight, serum hormone concentrations of FSH, LH and testosterone, and histological and cytological parameters returned to essentially normal values 52 days after injection. Zoladex was choosen for further experiments since a single injection previous to irradiation gives a more or less complete but absolutely reversible suppression of testicular function.

To obtain stem cell survival curves after irradiation both stem cell survival assays (repopulation index and number of haploid cells) were applied (chapter 9). Both assays demonstrated enhanced stem cell survival when the irradiation dose was split into fractions in comparison with the effect of a single dose. The observed fluctuations in the stem cell survival curve could be due to fluctuations in radiosensitivity of the stem cell possibly related to changes in the phase of the stem cell cycle. Studies on the effects of single versus fractionated irradiation on testicular stem cells have yielded conflicting results. Our results agreed with the observations of Withers et al. (64) and Lu et al. (41) that after a split dose irradiation no increase in stem cell radiosensitivity could be observed. The conflicting results may be attributed to the different methods for measuring stem cell survival (41) or to strain differences (48). Since the repopulation index for assessing stem cell survival was used, the possibility of different results due to different techniques was ruled out. Species differences could still be responsible for the differences. All previous studies were done with mice.

Much to our surprise testicular suppression with Zoladex and split dose irradiation made the stem cell even more vulnerable to gonadal irradiation damage. Hormonal suppression of spermatogenesis and irradiation led to an increase in stem cell depletion. This was also the case with a single dose of 6.0 Gy. From these findings we conclude that the relative increase in vulnerability has probably nothing to do with moving into a more sensitive phase of the stem cell cycle. The stem cells that had escaped destruction by X-rays, behaved as if no hormones were given. In other words, the fluctuations in radiosensitivity of stem cells appeared to be a rigidly regulated process, in which a hormonal drug apparently had no influence. This mechanism is very important and needs further investigation. We postulated that the surviving stem cells resumed their usual pre-irradiation steady-state proliferation pattern.

It seems that the administration of LH-RH analogues like Zoladex prior to irradiation does not give any improvement in the recovery of post treatment fertility. Our experiments indicate, however, a new therapeutic strategy for protection of fertility during

irradiation, not by hormonal suppression, but by using an 8 hour fractionation interval for radiotherapy instead of 24 hours as mostly performed. There is, as shown in this thesis, a relatively high vulnerability of stem cells if irradiated at 24 hours intervals. Of special interest for this hypothesis is the study of Deringer et al. (10), who described already in 1954 that mice regained fertility after being irradiated with 17.6 Gy at a dose rate of 0.088 Gy/ 8h day. Ruiter-Bootsma et al. (59) also observed an increase in the number of stem cells after an 8 hour fractionation interval. Further work is required to confirm this hypothesis.

Extrapolation of our results to man is difficult. Scanty information is available concerning the effects of testicular irradiation in the human (18,32,60,62). Spermatogenesis in man has been found (56) to be much more radiosensitive than in rodents: After an irradiation dose of 1.0 Gy, spermatogenesis will be disturbed for months. The difference between responses to irradiation could be attributed to differences in spermatogonial stem cell renewal between man and rodents (56). In man the isolated "As" stem cell has not been described up till now. Two morphologically and apparently functionally distinct spermatogonial cells have, however, been described (5,55): "Ad" (dark) and "Ap" (pale). The "Ad" cells are rounded cells with a spherical nucleus containing darkly-staining flakes of chromatin. The "Ad" cells do not proliferate in the normal testis and these cells are considered to be reserve stem cells. The "Ap" cells proliferate and differentiate regularly and have lightly staining chromatin. It has been found, in contrast to the data in rodents, that the numbers of "Ad" and "Ap" decreased very slowly during a period of several months after irradiation (56). Restoration of fertility is a function of stem cell survival and the kinetics of regeneration and repopulation. Differences in these kinetics of stem cell renewal could lead to interspecies or strain differences in radiation response (13,48). In man surviving stem cells after irradiation preferentially produce differentiating cells without repopulating themselves (56). If stem cells first would differentiate themselves

rather than repopulate, this would cause a further, secondary, depletion of spermatogonial reserves. However, if stem cells first repopulate themselves rather than differentiate, as is the case in rodents, this phenomenon would only result in a delayed recovery time (11,50). According to Meistrich et al. (44) the variation of this delay is not large. Of interest are the observations of Keulen et al. (36,37,38) that in the mouse enhanced stem cell renewal took place the first 15 days after cell loss caused by Myleran (an alkylating agent).

CONCLUSIONS

The results in this thesis lead to the conclusion that the effect of gonadal irradiation depends largely on the effects of irradiation on the relatively radioresistant stem cell. In rat testis the number of these stem cells were calculated to be approximately 24,000. Both radiosensitivity and the number of these stem cells were found to be comparable to those of mice. Direct quantification of stem cells with flow cytometry is not yet possible and quantification of spermatogonial kinetics with flow cytometry is doubtful. Using two stem cell-assays (number of haploid cells and the repopulation index) it became evident that no increased radiosensitivity occurred after split-dose irradiation. Both drugs tested gave a suppression of the pituitary-gonadal axis. Temporary interruption of the pituitary-gonadal axis by an LH-RH analogue (Zoladex) treatment had no protective effect on stem cells of the Wistar rat after split dose irradiation with time intervals ranging from 0 till 72 hours. Based on these findings it was concluded that the administration of an LH-RH analogue (Zoladex) prior to irradation effected no substantial improvement in the recovery of post treatment fertility. Several reasons can be given for the failure to preserve post irradation fertility. The most important reason is probably the fact that no increase in radiosensitivity after fractionated irradiation occurred and the fact that no change in stem cell behaviour was observed after hormonal treatment. We can not exclude that there may be a protective effect at higher irradiation doses or when the dose

is multifractionated. This certainly needs further investigation. Of clinical importance may be the fact that an 8 hour fractionation interval could result in less gonadal damage in reference to fertility.

From the foregoing it is evident that there is need for more search for a treatment which could reduce the gonadal damage as a side effect of irradation. Particular emphasis should be placed on the spermatogonial stem cells, because these stem cells play a crucial role after chemotherapy and irradiation in reference to fertility effects and little is known about the normal stem cell kinetics and the regulation of stem cell renewal. Special attention should also be given to the duration of the fractionation interval of irradiation. Our study suggests that an 8 hour fractionation interval in clinical radiation therapy could result in improvement of post treatment fertility.

References

1. Bain J and Keene J: Further evidence for inhibin· Change in serum luteinizing hormone and follicle-stimulating hormone levels after x-irradiation of rat testes. J. Endocr 66, 278-280, 1975.

2. Chowdhury AK: Thymidine-H^3 labeling of spermatogonia in rat and human seminiferous tubules mounted in toto. Anat. Rec. 169, 296, 1971.

3. Clausen OPF, Purvis K and Hansson V: Application of Micro-flow fluorometry to studies of meiosis in the male rat. Biol. Reprod. 17, 555-560, 1977.

4. Clausen OPF, Parvinen M and Kirkhus B: Stage-related variations in DNA fluorescence distribution during rat spermatogenic cycle measured by flow cytometry. Cytometry 6, 421-425, 1982.

5. Clermont Y : Renewal of spermatogonia in man. Am. J. Anat. 118, 509-524, 1966.

6. Clermont Y and Hermo L: Spermatogonial stem cells in the albino rat. Am. J. Anat. 142, 159-176, 1975.

7. Clow DJ and Gilette EL· Survival of type A spermatogonia following X-irradiation Rad. Res. 42, 397-404, 1970.

8. Cunningham GR and Huckins C Serum FSH, LH and Testosterone in Co^{60} γ irradiated male rats. Radiat. Res 76, 331-338, 1978.

9. Delic JI, Hendry JM, Morris ID and Shalet SM: Testicular endocrine injury in the adult rat after X-irradiation: Dose and time relationships. Proceedings of the seventh international congress of radiation research (ed. JJ Broerse et al , Martinus Nijhoff Publishers) Amsterdam C2-03, 1983.

10. Deringer MK, Heston WE and Lorenz E: Effects of long-continued total-body γ irradiation on mice, guinea pigs and rabbits, IV. Actions on the breeding behavior of mice in: R.E. Zirkle (Ed.), Biological effects of external X- and γ-Radiation, McGraw-Hill, New York, 149-168, 1954.

11. Erickson BH: Effect of Co^{60} γ radiation on the stem and differentiating spermatogonia of the post puberal rat. Radiat. Res. 68, 433-448, 1976.

12. Erickson BH: Survival and renewal of murine stem spermatogonia following Co^{60} γ radiation. Radiat. Res. 86, 34-51, 1981.

13. Erickson BH and Hall GG: Comparison of Stem-spermatogonial renewal and mitotic activity in the γ-irradiated mouse and rat. Mut. Res. 108, 317-335, 1983.

14. Glode LM, Robinson J and Gould SF: Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropropin-releasing hormone Lancet. I, 1132-1134, 1981.

15. Glode LM, Robinson J, Gould SF, Nett TM and Merril D: Protection of spermatogenesis during chemotherapy. Drugs Exptl. Clin. Res. 7, 367-378, 1982.

16. Hacker U, Schumann J and Gohde W: Effects of acute γ-irradiation on spermatogenesis as revealed by flow cytometry. Acta Rad. Oncol. 19, 361-368, 1980.

17. Hacker U, Schumann J, Gohde W and Muller K· Mammalian spermatogenesis as a biologic dosimeter for radiation. Acta Rad. Oncol. 20, 279-282, 1981.

18. Hahn EW, Feingold SM and Nisce L: Aspermia and recovery of spermatogenesis in cancer patients following incidental gonadal irradiation during treatment. Radiology 119, 223-225, 1976.

19. Hilscher W DNA synthesis; proliferation and regeneration of the spermatogonia in the rat. Arch. Anat. Microsc. 56, 75-84, 1967.

20. Hilscher W and Hilscher B: Autoradiographische Untersuchungen ueber die Kinetic der Spermatogenese der Wistar Ratte unter besonderer Berucksichtigung der Spermatocytogenese. Z. Zellforsch. 96, 625-640, 1969.

21. Hilscher W, Hilscher B and Makoski B: Histologische und Autoradiographische Untersuchungen zur "Praespermatogenese" und "Spermatogenese" der Ratte. Z. Zellforsch. 86, 327-350, 1968.

22. Hilscher B, Hilscher W and Maurer W: Autoradiographische Untersuchungen ueber den Modus der Prolifaeration und Regeneration des Samenepithels der Wistarratte. Z. Zellforsch. 94, 593-604, 1969.

23. Hopkinson CRN, Dulisch B, Gauss G, Hilscher W and Hirschlauser C: The effect of local testicular irradiation on testicular histology and plasma hormone levels in the male rat. Acta Endocr. 87, 413-423, 1978.

24. Huckins C, Kopriwa BM: A technique for the radioautography of germ cells in whole mounts of seminiferous tubules. J. Histochem. Cytochem. 17, 848-852, 1969.

25. Huckins C: Cell cycle properties of differentiating spermatogonia in adult Spraque-Dawley rats. Cell Tiss. Kinet. 4, 139-154, 1971.

26. Huckins C: The spermatogonial stem cell population in adult rats I Their morphology, proliferation and maturation. Anat. Rec. 169, 533-558, 1971.

27. Huckins C: The spermatogonial stem cell population in adult rats II a radioautographic analysis of their cell cycle properties. Cell Tiss. Kinet. 4, 313-334, 1971.

28. Huckins C: The spermatogonial stem cell population in adult rats III Evidence for a long-cycling population. Cell Tiss. Kinet. 4, 335-349, 1971.

29. Huckins C: The morphology and kinetics of spermatogonial degeneration in normal adult rats: An analysis using a simplified classification of the germinal epithelium. Anat. Rec. 190, 905-926, 1978.

30. Huckins C and Oakberg EF: Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II The irradiated testes. Anat. Rec. 192, 529-542, 1978.

31. Huckins C: Behavior of stem cell spermatogonia in the adult rat irradiated testis. Biol. Reprod. 19, 747-760, 1978.

32. Jackson HL, Hass AC, Sooby D and Marschke CH: The gonadal exposure of boys and young men treated with inverted y-fields: Its reduction and genetic significance. Radiology 96, 181-186, 1970.

33. Jarell J, Younglai EV, Barr R, O'Connel G, Belbeck L and McMahon A: The protection of ovarian follicles from ionizing radiation with agonist LHRH ethylamide. (Abstract) Presented at

the 67 th Annual Meeting of the Endocrine Society, Baltimore, Maryland, U.S A. 1985

34. Jong de FH and Sharpe RM: Gonadotrophins, testosterone and spermatogenesis in neonatally irradiated male rats Evidence for a role of the sertoli cell in follicle-stimulating hormone feedback J. Endocr. 75, 209-219, 1977.

35 Johnson DH, Linde R, Hainsworth JD, Vale W, Rivier J, Stein R, Flexner J, Van Welch R and Greco A· Effect of a luteinizing hormone releasing hormone agonist given during combination chemotherapy on posttherapy fertility in male patients with lymphoma Preliminary observations. Blood 65, 832-836, 1985.

36. Keulen van CJG and de Rooij DG· Spermatogonial stem cell renewal in the mouse. II. After cell loss. Cell Tiss. Kinet 6, 337-345, 1973

37. Keulen van CJG and de Rooij DG. The recovery from various gradiations of cell loss in the mouse seminiferous epithelium and its implications for the spermatogonial stem cell renewal theory Cell Tissue Kinet 7, 549-558, 1974.

38. Keulen van CJG and de Rooij DG: Spermatogenic clones developing from repopulating stem cells surviving a high doses of an alkylating agent. I First 15 days after injury Cell Tiss. Kinet. 8, 543-551, 1975.

39. Kramer MF, Davids JAG and van der Ven THPA: Effect of 1 MeV fast-neutron irradiation on spermatogonial proliferation in mice. Influence of dose fractionation with different intervals. Int. J. Radiat. Biol 25, 253-260, 1974.

40. Libbus BL, Schuetz AW· Analysis of the progression of meiosis in dispersed rat testicular cells by flow cytofluorometry. Cell Tiss. Kinet. 11, 377-391, 1978.

41. Lu CC, Meistrich ML and Thames HD Survival of mouse testicular stem cells after ɣ or neutron irradiation. Radiat Res. 81, 402-415, 1980.

42. Lushbaugh CC and Casarett GW The effects of gonadal irradiation in clinical radiation therapy: A review. Cancer 37, 1111-1120, 1976.

43. Matus M, Nicosia SV and Meadows AT Gonadal effects of cancer therapy in boys. Cancer 55, 2353-2363, 1985.

44. Meistrich ML, Hunter NR, Suzuki N, Trostle PK and Withers HR: Gradual regeneration of mouse testicular stem cells after exposure to ionizing radiation Radiat. Res. 74, 349-362, 1978.

45. Meistrich ML, Lake S, Steinmetz LL and Gledhill BL· Flow cytometry of DNA in mouse sperm and testis nuclei. Mut. Res. 49, 383-396, 1978.

46. Meistrich ML, Lake S, Steinmetz LL and Gledhill BL: Increased variability in nuclear DNA content of testis cells and spermatozoa from mice with irregular meiotic segregation. Mut. Res. 49, 397-405, 1978.

47. Meistrich ML: Quantitative correlation between testicular stem cell survival, sperm production and fertility in the mouse after treatment with different cytotoxic agents. J. Androl. 3, 58-68, 1982.

48. Meistrich ML, Finch M and Lu CC: Strain differences in the response of mouse testicular stem cells to fractionated radiation. Radiat. Res. 97, 478-487, 1984.

49. Monesi V: Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. J. Cell Biol. 14, 1-18, 1962.

50. Oakberg EF: Initial depletion and subsequent recovery of spermatogonia of the mouse after 20 R of ɣ rays and 100, 300, and 600 R of X-rays. Radiat. Res. 11, 700-719, 1959.

51. Oakberg EF and Huckins C: Spermatogonial stem cell renewal in the mouse as revealed by H^3-thymidine labeling and irradiation. J. Endocr. 60, 287-293, 1974.

52. Oakberg EF: Effects of radiation on the testis. In Handbook of Physiology Section 7. Endocrinology Vol. V (ed. Greep RO et al. Am. Phys. Soc. Washington), 233-243, 1975.

53. Oakberg EF: Differential spermatogonial stem cell survival and mutation frequency. Mut. Res. 50, 327-340, 1978.

54. Pfitzer P, Gilbert P, Rolz G and Vyska K: Flow cytometry of human testicular tissue. Cytometry 3, 116-122, 1982.

55. Rodriquez-Rigau LJ, Zukerman Z, Weiss KD and Steinberger E: Hormonal control of spermatogenesis in man:comparisons with the rat. In Testicular Development, Structure and function, ed. by A. Steinberger and E. Steinberger, Raven Press, New York, 139-146, 1980.

56. Rowley MJ, Leach DR, Warner GA and Heller CG: Effect of graded doses of ionizing radiation on the human testes. Radiat. Res. 59, 665-678, 1974.

57. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Response of stem cells in the mouse testes to fission neutrons of 1 MeV mean energy of 300 KV X-rays. Methodology, dose-response studies, relative biological effectiveness. Radiat. Res. 67, 56-68, 1976.

58. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean

energy or 300 KV X-rays. Radiat. Res. 71, 579-592, 1977.

59. Ruiter-Bootsma de AL, Kramer MF, de Rooij DG and Davids JAG: Survival of spermatogonial stem cells in the mouse after split-dose irradiation with fission neutrons of 1-MeV mean energy. Effect of the fractionation interval. Radiat. Res. 79, 289-297, 1979.

60. Sandeman,T.F.θ effects of X-irradiation on male human fertility. Brit. J. Radiol. 39, 901-907, 1966.

61. Sheridan W: The effects of the time interval in fractionated X-ray treatment of mouse spermatogonia. Mut. Res. 13, 163-169, 1971.

62. Speiser B, Rubin P and Casarett G: Aspermia following lower truncal irradiation in Hodgkin's disease. Cancer 32, 692-698, 1973.

63. Verjans HL and Eik-Nes KB: Hypothalamic-pituitary-testicular system following testicular X-irradiation. Acta Endocr. 83, 190-200, 1976.

64. Withers HR, Hunter N, Barkley HT and Reid BO: Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testes. Radiat. Res. 57, 88-103, 1974.

SUMMARY

The success of radiotherapy and chemotherapy has prolonged the survival of cancer patients. However, both radiotherapy and chemotherapy have side-effects of which gonadal dysfunction is one. This iatrogenic side-effect plays a considerable role in the life of patients in the child bearing age. Recent studies in murine have shown that the gonads can be protected from the effects of chemotherapy by suppression of the pituitary-gonadal axis. We hypothesized that the male gonads could also be protected by hormonal suppression of spermatogenesis during irradiation. It is known that dividing cells are more radiosensitive then non-dividing cells Reducing the rate of spermatogenesis by hormones could bring the testis into a resting state comparable to prepuberty and in this way it could be possible to prevent cell division and at least a part of the damage by subsequent irradiation. To test this, a rat model was investigated, where hormonal suppression of spermatogenesis during irradiation was achieved and stem cell survival was measured. Attention was focussed on the stem cell, because this cell is primarily responsible for the late effects of radiation on fertility. Flow cytometrical and histological techniques were used as parameters for measuring stem cell survival. Serum concentrations of FSH, LH and testosterone were measured to evaluate the hormonal suppression.

The role of flow cytometry in measuring spermatogonial and stem cell kinetics was evaluated by a computer simulation model. Direct quantification of stem- or spermatogonial cell kinetics appeared to be impossible with this technique. Using two-parameter DNA analysis (DNA content and the cell size) we were able to distinguish non-germ cells from germ cells. Concerning spermatogonial and stem cell proliferation no extra information was obtained. Because there is a relation between stem cell survival and the number of spermatids, the number of haploid cells (=spermatids) as measured by flow cytometry was used as an indirect stem cell assay. This was used in combination with the repopulation index, which has been shown in the literature to correlate very well with stem cell survival. The

radiosensitivity of the stem cells was measured using both stem cell assays. A Do value of 2.33 Gy (repopulation index) and 2.08 Gy (number of haploid cells) was obtained. An estimation of the number of stem cells was calculated by the computer model to be around 21,000 and 28,000 per testis. Two hormonal substances (Danazol® and Zoladex®) were used to produce temporary interruption of the pituitary-gonadal axis. Both drugs have been shown to suppress effectively the pituitary-gonadal axis. The drug Zoladex® was choosen for further experiments, because the hormonal suppression appeared to be almost completely reversible and the method of administration has practical advantages. Local testicular irradiation was either given as a single dose or split into fractions. Conflicting results have been reported in the literature about stem cell survival after fractionated irradiation. Our results showed that after fractionated irradiation with time intervals ranging from 0 till 72 hours stem cell survival was higher in comparison with the single irradiation dose. However, stem cell survival appeared to be of no constant value, but showed time dependent fluctuations with a minimum of survival at 24 hours and a maximum of survival at 8 hour irradiation time interval. This suggested that an 8 hour fractionation interval could result in less gonadal damage in reference to fertility effects in comparison with the 24 hour fractionation interval. The combination of hormonal suppression of spermatogenesis by Zoladex® and fractionated irradiation showed no protective effect on stem cells in rats. Contrarily, this combination made the stem cell even more vulnerable to gonadal irradiation damage and considerable less stem cell survival was observed. Based on these findings it was evident that the administration of Zoladex® prior to irradiation effected no substantial improvement in the recovery of post treatment fertility in rats. Our study in rats indicated that improvement in fertility after irradiation could be achieved by an irradiation fractionation interval of 8 hour. Extrapolation of these findings to man is difficult, but could be of clinical importance. More research is needed to evaluate this finding for clinical use.

SAMENVATTING

Bij de behandeling van oncologische patienten is grote vooruitgang geboekt door zowel radiotherapie als chemotherapie. Beide behandelingsmethoden veroorzaken echter ook bijwerkingen, waarvan gonadale dysfunctie er een is. De gonadale beschadiging kan uitmonden in een lange periode van infertiliteit of zelfs steriliteit. Vooral voor jonge patienten kan dit van groot belang zijn. In recente dierstudies is aangetoond, dat door onderbreking van de hypofysaire-gonadale as met behulp van hormonen, het beschadigend effect van chemotherapie op de gonaden aanmerkelijk minder kan zijn. Analoog met deze bevindingen zou het volgens ons ook mogelijk moeten zijn door hormonale suppressie van de spermatogenese tijdens bestralingen een bescherming van de gonaden te bewerkstelligen. Het is bekend dat delende cellen veel stralingsgevoeliger zijn dan cellen in de rustfase. Delende stamcellen kunnen dan ook makkelijk door een tweede bestraling vernietigd worden. Gefractioneerde bestraling zou op deze manier leiden tot een verhoogd stamcel verlies, hetgeen steriliteit tot gevolg kan hebben. In dit proefschrift is getracht aan de hand van dierexperimenteel onderzoek na te gaan of door middel van hormonale suppressie van de spermatogenese de nadelige effecten van radiotherapie op de gonaden kunnen worden gereduceerd. Met name werd de aandacht gelegd bij de stamcel, omdat deze een cruciale rol speelt met betrekking tot de latere vruchtbaarheid na bestraling. Flow cytometrische technieken en histologische quantificatie werden gebruikt als parameters voor stamcel overleving. Tevens werden de hormoon spiegels van FSH, LH en testosteron bepaald in serum om de hormonale suppressie te evalueren.

Met behulp van een computer simulatie model werd bekeken of het mogelijk was om met flow cytometrische technieken de kinetiek van de stamcel en spermatogonia te quantificeren. Dit bleek, ook na het gebruik van 2-parameter DNA analysis (DNA inhoud en grootte van de cel), niet mogelijk te zijn. Wel bleek het mogelijk om cellen behorend bij de spermatogenese en die er niet toe behoren, te onderscheiden. Omdat er een relatie is tussen het aantal

spermatiden en het aantal stamcellen kan een indirecte maat voor het aantal stamcellen verkregen worden, door het aantal haploide cellen (=spermatiden) flow-cytometrisch te meten. Deze parameter werd in combinatie gebruikt met een histologische stamcel assay (repopulatie index). De stamcel stralingsgevoeligheid werd met beide assays bepaald. Er werd een Do waarde berekend van 2.33 Gy (repopulatie index) en 2.08 Gy (aantal haploide cellen). Het aantal stam cellen werd met behulp van het computer model berekend op 21,000 tot 28,000 per testis. Twee hormonen (Danazol® en Zoladex®) werden bij ratten getest op hun remmend effect op de spermatogenese. Zowel Danazol® als Zoladex® gaven een suppressie van de spermatogenese. De experimenten werden voortgezet met Zoladex®, omdat dit hormoon een reversibele werking en ook practische voordelen had. Bestraling werd zowel eenmalig als gefractioneerd gegeven. De literatuur geeft verschillende resultaten te zien wat betreft de stamcel overleving na gefractioneerde bestralingen. Uit onze resultaten konden we concluderen, dat na gefractioneerde bestralingen geen verhoogd stamcel verlies optrad. De stralingsgevoeligheid van de stamcel vertoonde wel tijdsafhankelijke fluctuaties. Het stamcel verlies was het grootst als de tijdsduur tussen de bestraling 24 uur bedroeg en het kleinst bij een bestralingsinterval van 8 uur. De combinatie van hormonale suppressie (Zoladex®) van de spermatogenese en gonadale bestraling, resulteerde in een aanzienlijk stamcel verlies. De resultaten in deze dierstudie suggereren, dat hormonale suppressie met Zoladex®, uiteindelijk niet zal resulteren in een verbetering van de fertiliteit na bestraling. Verbetering met betrekking tot latere vruchtbaarheid zou bereikt kunnen worden, wanneer het tijdsinterval van bestralingen op 8 uur zou worden gesteld. Extrapolatie van deze resultaten naar de mens is moeilijk en klinisch onderzoek is nodig om te evalueren of gefractioneerde bestraling met een tijdsinterval van 8 uur inderdaad leidt tot een verbetering van de fertiliteit.

ACKNOWLEDGEMENT

I would like to express special gratitude to Prof. Dr. I. Kazem under whose direction this work was initiated and who introduced to me the fields of biomedical research. His work is deeply appreciated.

I am very grateful to Mr. H.J. Beck for his enthusiastic cooperation and many good ideas and to Drs. J.W. Scholtz for his interest and his accuracy in microscopical counting of cells. Thanks are due to Dr. U. Hacker of the department of Radiobiology of the University of Münster, W. Germany, for her interest and valuable discussions; to Dr. D.G. de Rooij of the department of Cyto-Histology of the University of Utrecht for advice and his hospitality and to Dr. J. van Egmond for helping to prepare the lay-out of the manuscript. Finally I am obliged to all persons who assisted and supported me during these studies.

I acknowledge the collaboration with the following departments:

Central Animal Laboratory (Dr. W.J.I. van der Gulden): Special credits must go to Mr. J. Koedam, Mr. H.P.N. van Wezel and Mr. W.M. Smits for taking care of the animals and for their excellent technical assistance. Their help during the irradiation procedures (often in the evening hours) is greatly appreciated.

Department of Pathology (Prof. Dr. G.P. Vooys): Special thanks are due to Mrs. O. Wolf and Mr. L.J.M. Burgers for preparing light microscopial sections.

Department of Obstetrics and Gynaecology: Dr. C.M.G. Thomas and Ir. M.F.G. Segers for measuring serum hormone concentrations of FSH, LH and testosterone.

Department of Clinical Endocrinology (Prof. Dr. T.J. Benraad): Mr. J. Hofman for measuring serum testosterone levels.

Department of Pharmacology (Prof. Dr. E. van der Kleijn): Drs. H. Bakker and Mrs. C. Stoel for advice and for preparing the drugs.

Department of Nuclear Medicine (Dr. F.H.M. Corstens): Thanks are due to Drs. W.C.A.M. Buijs for statistical advice and Mr. E. Koenders for helping to prepare cell suspensions.

Department of Medical Photography (Mr. A.T.A. Reynen).

Danazol was kindly supplied by Sterling-Winthrop Research Institute, London, U.K. and Zoladex by ICI Ltd., Pharmaceuticals Division, Macclesfield, Cheshire, U.K.

CURRICULUM VITAE

1953 : Born at Heerlen, The Netherlands on February 2nd.

1973 : Secondary school (Atheneum-B) at Sint Bernardinus College at Heerlen.

1973 : Medical study at the University of Nijmegen.

1976 : Research fellow at the Department of Physiology (Prof. Dr. M. Tenney), at Dartmouth Medical School Hanover, New Hampshire, U.S.A.

1976 : Student assistant at the Department of Biology (Dr. J.A.M. van Kemenade) at the University of Nijmegen.

1980 : As a part of the medical study: work with a general practioner (Dr. D.S. Browne) at Brockenhurst, New Hampshire, England.

1980 : Medical Doctor degree.

1980 : Trainee at the Sint Joseph Hospital at Veghel (Director: Drs. W.A.M. Driessen), working at the Department of Surgery, Internal Medicine and Gynaecology and Obstetrics.

1981 : Start of training in Nuclear Medicine, Department of Radiotherapy and Nuclear Medicine (Prof. Dr. I. Kazem) at the University of Nijmegen.

1986 : Registered in Nuclear Medicine.

STELLINGEN

Stellingen behorende bij het proefschrift van M.J.P.G. van Kroonenburgh: Hormonal protection of spermatogenic stem cells during irradiation (A histological, hormonal and flow cytometric study in rats). Nijmegen, 1986.

I

Indien groepen patienten met carcinoom vergeleken worden volgens de TNM classificatie, dient men rekening te houden met het Will-Rogers fenomeen: When the Okies left Oklahoma and moved to California, they raised the average intelligence level in both states.

N. Eng. J. Med. 312, 164, 1985.

II

Bij Hodgkin patienten verdient het aanbeveling, zolang er nog geen goede methode ter beschikking staat ter bescherming van de fertiliteit, het zaad zo spoedig mogelijk in te laten vriezen, voordat met therapie begonnen wordt.

III

Onderzoek naar de invloed van het bestralingsinterval op de vruchtbaarheid bij de man dient hoge prioriteit te hebben.

IV

Het is voor alsnog niet mogelijk om met behulp van de flow cytometrie betrouwbare veranderingen in de kinetiek van de spermatogonien te meten.

V

Bij een stoplicht wordt pas duidelijk dat veel meer mensen kleurenblind zijn dan tot nu toe werd aangenomen.

VI

Niet betere apparatuur, maar betere samenwerking met mensen leidt tot betere resultaten.

VII

Tsjernobyl is geen dode waard.

VIII

Om voor elke infertiliteits patiente met tuba pathologie tot een juiste indicatie stelling te komen, dient in-vitro-fertilisatie uitsluitend te worden verricht in een centrum waar ook een grote ervaring in tuba chirurgie bestaat.

IX

Het therapeutisch gebruik van gelabelde monoclonale antilichamen tegen maligne aandoeningen behoort nog steeds tot het rijk van de magic bullets.

X

Het in het openbaar verdedigen van een proefschrift is eerder een vorm van vermaak dan dat het de vooruitgang van de wetenschap ten goede komt.