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# EFFECTS OF RADIATION ON MEIOTIC SYSTEMS

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# MAMMALIAN GAMETOGENESIS AND SPECIES COMPARISONS IN RADIATION RESPONSE OF THE GONADS*

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**Abstract**

MAMMALIAN GAMETOGENESIS AND SPECIES COMPARISONS IN RADIATION RESPONSE OF THE GONADS. Primordial germ cells have an extra-gonadal origin and migrate to the genital ridge early in embryonic development. After a series of mitoses, they transform into oocytes in the female and into type A spermatogonia in the male.

In females, the total supply of germ cells is present either before or shortly after birth; development of the oocyte is arrested in diplotene of meiotic prophase. The nuclear characteristics of the 'arrested' diplotene varies greatly between species, and is an important factor in species differences in ovarian radiation response. In adults of all mammals, the radiation response of the ovary involves a fixed population of primary oocytes. Oogonia are absent; any loss from the oocyte pool cannot be replaced. Type A spermatogonia persist throughout the reproductive life span of the male, and a complete array of gametogenic stages is present in the adult. Radiation resistance of spermatids and sperm results in an initial period of post-irradiation fertility; destruction of spermatogonia and possibly spermatocytes leads to oligospermia with doses of several hundred R; fertility eventually returns owing to repopulation of the seminiferous epithelium [illegible] few surviving type A spermatogonia. The different radiation response of males and females therefore [illegible] attributed to basic differences in gametogenesis and the array of germ cell stages present in the [illegible] gonad. A radiation response typical for all males also occurs in man. However, exact comparison of radiation sensitivity in man and experimental animals is not possible owing to differences in the control of spermatogonial differentiation which results in species differences in both initial depletion and subsequent recovery of the spermatogonial population.

## INTRODUCTION

Our understanding of the radiation response of the gonads is dependent on our knowledge of the origin of the germ cells, their development during uterine and pre-pubertal life, and gametogenesis in the adult. Wherever possible, our meager data on man have been included; but for the most part, the formulation of basic principles of normal gametogenesis, and especially of radiation response, has been based on data on experimental animals.

The most striking difference in gametogenesis in mammals is that which occurs between the sexes. In the male, stem cells (spermatogonia) and continued formation of mature gametes occur throughout the reproductive life-span; whereas inthe female, the definitive supply of oocytes is formed either before or shortly after birth. Oogonia are not present in the adult ovary. As a result of these fundamental differences in gametogenesis, the radiation response of males differs greatly from that of the female.

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## GAMETOGENESIS

### Prenatal and neonatal

Migration of primordial germ cells from an extragonadal origin to the germinal ridges was first observed in human embryos [1]. Later, Chiquoine [2] demonstrated selective alkaline phosphate positive staining of the primordial germ cells of the mouse, and by this technique was able to trace their migration from their origin in the region of the caudal end of the primitive streak, root of the allantoic mesoderm, and yolk sac splanchnopleure in the 8-day mouse embryo, to the germinal ridges of the 12-day embryo. The demonstration by Mintz [3] that sterility and reduced fertility associated with certain genotypes of the mouse can be correlated with a reduced number of primordial germ cells is conclusive evidence that these are the stem cells of the definitive gametes. Oocyte counts in rat embryos have led to the same conclusion [4]. Mitotic division occurs both during migration of the primordial germ cells and in the germinal ridge. Primordial germ cells transform directly into oocytes in the female [3-6]. In males, they give rise to type A spermatogonia soon after birth in the rat [7], and by 10 years in man [8]. Although Charny et al. [8] did not use the precise system of cell classification given by Clermont [9], their data indicate that the basic developmental pattern observed in other mammals also occurs in man.

### Adult male

Our understanding of normal spermatogenesis has advanced markedly in recent years owing to the accurate identification of both cells and developmental sequences made possibly by the periodic acid-Schiff technique [9, 10, 11]. In rodents, stages of the cycle of the seminiferous epithelium occupy relatively large areas of the tubule, and a given tubule cross section usually shows a single stage. In man, however, an average of three different stages appears in a single cross section, and while analysis of the cycle of the seminiferous epithelium is therefore very difficult, it has been shown to be fundamentally the same as that observed in other species [12].

Type A spermatogonia are the stem cells of the mammalian testis. By the process of stem cell renewal, the type A population maintains a steady state while giving rise to an unlimited number of differentiated spermatogonial types, which develop into sperm. The following brief description for the mouse is similar to that of Clermont [9] for the rat, and though species differences occur, the same basic process has been observed in guinea pigs, monkeys, and man. Four divisions of type A spermatogonia occur [13]. At the conclusion of the fourth division, most cells transform into intermediate spermatogonia, with morphological characteristics intermediate between those of type A and type B. The intermediate spermatogonia divide to form spermatogonia of type B, which in turn divide to form resting primary spermatocytes. A few type A spermatogonia fail to differentiate, and form the stem cells for the next multiplicative cycle. In the mouse and rat, either four or five cell generations, depending on stage of the cycle of the seminiferous epithelium, occur in a given tubule section. Both the cellular association and number of cells are characteristic for each stage of the cycle; thus quantitation of radiation response of specific cell types is possible. The above model needs to be modified only slightly for man, where two types of type A cells occur, A dark (Ad), and A pale (Ap) [12]. Ad spermatogonia differentiate into Ap, and Ap divide to form spermatogonia B. From there the process is similar, with

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permiogenesis involving changes leading to development of characteristic sperm morphology of each species.

The time required for type A spermatogonia to develop into spermatozoa appears to be a biological constant for each species, and is unaffected by radiation [14] or hormone levels [15, 16] even though both of these procedures can result in severe depletion of the seminiferous epithelium. Estimates of the duration of spermatogenesis are about 35 days in the mouse [17], 49 days in the ram [18], 48 days in the rat [19], and 72-74 days in man [16]. These estimates, together with information on the dynamics of cell populations and relative frequency of stages of the cycle of the seminiferous epithelium, make possible the calculation of the life-span of each cell type. Such calculations, as given for mouse and man in Table I, are invaluable in studying the radiation response of specific cell types, and in comparing the radiation response of different species.

### Adult females

The most distinctive feature of oogenesis in mammals is the absence of stem cells (oogonia) from the adult ovary [20]. All oogonia enter meiosis either before or shortly after birth, and usually develop as far as diplotene. Nuclear morphology of the diplotene stage of the "arrested" oocyte, however, varies widely between species; a "typical" diplotene is characteristic of the human, goat, and dog; a synizesis-like diplotene is characteristic of the guinea pig; and a diffuse, interphase-like diplotene (dictyate) is present in mouse and rat [21]. In all species, the chromosomes become diffuse and oxyphilic during growth of the follicle, and probably are in a lampbrush stage. The first meiotic division occurs in the ovary; ovulation occurs about 6 h later with the chromosomes in metaphase of the second r division [22, 23].

Since the female has a fixed pool of oocytes established early in life, it would be expected that the number of oocytes would decrease with age owing to follicular atresia and ovulation. This decrease has indeed been observed [24, 25] and constitutes a cogent argument against formation of oocytes in the adult mammalian ovary [24]. Furthermore, in the mouse, DNA synthesis typical of preleptotene occurs only in utero [26, 27], and oocytes labeled in utero persist in the adult ovary [27]. Finally, genetic factors which reduce fertility also reduce the number of primordial germ cells in the embryonic gonad [3]. As a result of the above observations, the direct relationship between primordial and definitive germ cells is now firmly established.

## RADIATION RESPONSE

### Females

The radiation response of the adult ovary, therefore, will be limited to diplotene and later meiotic stages in the adult; irradiation during embryonic, fetal, and neonatal development will have to be used in study of stages from primordial germ cells through pachytene. Whereas all female germ cells go through a similar developmental process, the correlation of germ cell stage with post-conception age, synchrony of the cell population, and stage at which oocyte development is arrested vary between species; thus, even though specific cells may have a similar radiation response wherever they occur, large species differences in radiation response of the ovary are to be expected.

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Table I. Timing of cell development in spermatogenesis of mouse and man

| Mouse | | | Man* | | |
|---|---|---|---|---|---|
| Cell type | Duration (days) | Interval to release of mature sperm from testis (days) | Cell type | Duration (days) | Interval to release of mature sperm from testis (days) |
| Spermatogonia | | | Spermatogonia | | |
| A | always present | 33-36 | Ad | always present | 72-74 |
| Intermediate | 1.1 | 29-30 | Ap | 16.0 | 73-57 |
| B | 1.2 | 28-29 | B | 11.2 | 46-57 |
| Primary Spermatocytes | | | Primary Spermatocytes | | |
| Preleptotene | 1.3 | 26-28 | Preleptotene | 1.0 | 45-46 |
| Leptotene | 1.3 | 25-26 | Leptotene | 3.7 | 41-45 |
| Zygotene | 1.6 | 23-25 | Zygotene | 2.9 | 38-41 |
| Pachytene | 7.3 | 16-23 | Pachytene | 15.5 | 23-38 |
| Diplotene | 0.9 | 15-16 | Diplotene | 0.8 | 22-23 |
| Meiotic divisions | 0.4 | 15 | Meiotic divisions | | |
| Spermatocyte II | 0.4 | 14-15 | Spermatocyte II | | |
| Spermatids | 14.0 | 0-14 | Spermatids | 22.3 | 0-22 |

* Computed from data of Heller and Clermont (see ref. 16).

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Table II. Mean numbers of oocytes surviving 50 R in the m. .nd 200 R in the guinea pig (From Oakberg and Clark; see ref 21)

| Time after irradiation (days) | Early follicles | | | | Late follicles | | | |
|---|---|---|---|---|---|---|---|---|
| | Mice (50 R) | | Guinea pigs (200 R) | | Mice (50 R) | | Guinea pigs (200 R) | |
| | No. cells* | Exptl./Control | No. cells | Exptl./Control | No. cells* | Exptl./Control | No. cells | Exptl./Control |
| Control | 3,549 | | 33,720** | | 46 | | 35** | |
| 1 | 959 | 0.270 | 37,780 | 1.120 | 48 | 1.043 | 15 | 0.429 |
| 2 | 27 | 0.008 | 21,020 | 0.623 | 55 | 1.196 | 3 | 0.086 |
| 3 | 35 | 0.010 | 17,580 | 0.521 | 61 | 1.326 | 0 | 0.000 |
| 7 | | | 15,420 | 0.457 | | | 2 | 0.057 |
| 10 | 24 | .007 | 13,300 | 0.394 | 65 | 1.413 | 0 | 0.000 |
| 14 | | | 11,560 | 0.343 | | | 41 | 1.171 |
| 20 | 10 | .003 | | | 71 | 1.543 | | |
| 21 | | | 18,440 | 0.547 | | | 49 | 1.400 |
| 42 | | | 10,500 | 0.311 | | | 19 | 0.543 |
| 45 | 3 | .001 | | | 10 | 0.217 | | |

* All means for mice based on three animals per group.

** Means for guinea pigs based on three controls; data based on one animal for each time after irradiation.

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Table III. Effect of meiotic prophase stage on survival and on chromosome breakage in primary spermatocytes of the mouse

| Meiotic prophase stage | Survival data | | | Chromosome breakage $D_{50}$ | |
|---|---|---|---|---|---|
| | Point estimate $LD_{50}$ (R) | 95% confidence limits | | Anaphase I | Anaphase II |
| | | Lower | Upper | | |
| Preleptotene | 205 | 175 | 233 | 612 ± 93 | |
| Leptotene | 492 | 273 | 719 | 1443 ± 182 | |
| Zygotene | 520 | 413 | 637 | 411 | |
| Pachytene (early) | 404 | 329 | 470 | 242 | |
| Pachytene (mid) | 382 | 301 | 452 | 265 | |
| Pachytene (late) | 664 | 601 | 733 | 321 | |
| Diplotene | 564 | 365 | 771 | 275 | |
| Diakinesis - Metaphase I | 837 | 679 | 1161 | | |
| Diakinesis | | | | 204 | |
| Metaphase I | | | | | 138 ± 6 |

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In the mouse, 50 R of x-rays reduces the number of litters to four per female, with permanent sterility thereafter [28]. This can be attributed to killing of the small oocytes, and subsequent failure to replace the older, more resistant stages as they mature and are ovulated [29, 30]. A similar response is observed in the rat [31], but higher doses are required to produce comparable effects. The response in guinea pigs is quite different, however, and a comparison of survival of early and late follicle stages in mice and guinea pigs is given in Table II. In the guinea pig, small oocytes are resistant and growing oocytes sensitive, which is the reverse situation of that in the mouse. As a result, fertility of guinea pigs is unaffected by acute x-ray exposures of several hundred R. Likewise, fertility of dogs is normal after 300 R x-ray exposures [32], rabbits show fertility for a considerable time after irradiation [33], and women have conceived a year or more after exposures of 300-400 R, with presumed normal fertility thereafter [34, 35].

The reasons for species differences in fertility of irradiated females are not well understood, but normal reproductive life-span, normal rate of oocyte depletion, frequency distribution of oocyte stages with different radiation sensitivity, and nuclear characteristics of the "arrested" oocyte all probably are important factors. Differences between species in nuclear cytology of the oocyte have been observed [21], and though we do not understand the basic mechanisms involved, a correlation exists between chromosome appearance and radiation response. High sensitivity to cell-killing is a characteristic of the dictyate nuclei of mouse and rat, which makes these species poor models for generalizations on adult ovarian radiation response in other mammals. However, periods of high sensitivity may occur during prenatal or neonatal development in species where the adult ovary is relatively resistant, and extreme caution should be used in irradiation exposure at this time. The most important fact is that the ovaries of different species show widely divergent radiation response.

## Males

Recent advances in our understanding of the normal dynamics of spermatogenesis has made possible the evaluation of the cellular responses in both depletion and regneration of the seminiferous epithelium. Spermatids show no morphological or gross functional damage (sensitivity to genetic damage is fairly high, however) even after doses of 1000 R or more, complete spermiogenesis, and give rise to the period of initial fertility following doses of several hundred R [36]. After irradiation, the frequency of cells degenerating during meiotic division, and the frequency of bridges and fragments at anaphase are increased. The result is depletion in the number of spermatids formed, and an increase in the proportion of spermatids with various degrees of heteroploidy. A measure of comparative sensitivity of different meiotic prophase stages to degeneration during meiotic division is shown in Table III, and varies from an $LD_{50}$ of 205 R for preleptotene to 837 R for diakinesis-metaphase I. Frequency of abnormal anaphases resulting from chromosome breakage, translocation, and formation of acentric fragments also are given in Table II. The ranking of sensitivities is almost the reverse of that for degeneration, with diakinesis-metaphase I most sensitive and leptotene least sensitive. As a result of degeneration and gross chromosomal abnormality, contribution of spermatocytes to post-irradiation gamete production is dose-dependent.

Since spermatocytes and spermatids have a relatively short duration in comparison to reproductive life-span, spermatogonia are the most important cells in radiation response of the mammalian testis. Killing of differentiating spermatogonia leads to maturation depletion of the testis, azoospermia, and the temporary period

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of infertility characteristic of males given a single radiation exposure. A few type A spermatogonia survive, however, and repopulate the seminiferous epithelium [37]. Depletion and recovery of apparently normal type A spermatogonia in the mouse are shown for a wide range of doses in Fig. 1. The time of maximum depression of cell numbers is dose-dependent owing to initiation of division among surviving cells before the process of cell degeneration has been completed. The interaction of these two processes is characteristic for each dose, and there is no optimum time for comparing the effects of all the doses given. As would be expected, recovery is more rapid after low doses, but a significant increase in the spermatogonial population has occurred by 10 days even after 600 R. Eventually, the entire array of spermatogenic cells is regenerated.

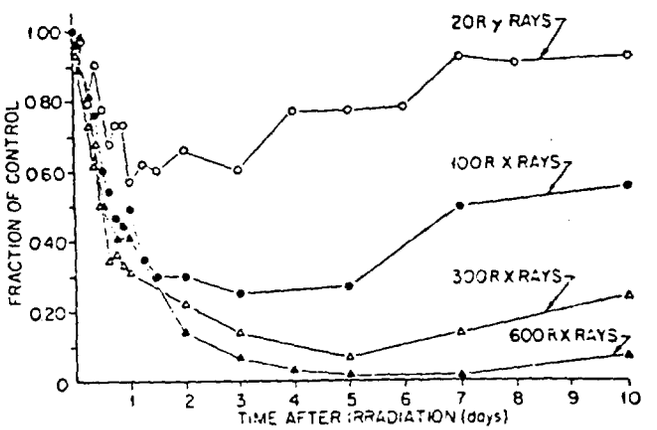


FIG. 1. Experimental/control ratios for 'normal' type A spermatogonia of the mouse 1 h to 10 days after irradiation.

Human testis

In man, the Ad spermatogonia are thought to be the stem cells of the seminiferous epithelium [12, 16]. Radiation response of the Ad spermatogonia to local x-ray exposure of the testis, as measured by number of normal cells, is shown in Fig. 2. (These data are based on slides provided by Dr. Carl G. Heller, Pacific Northwest Research Foundation, Seattle, Washington.) The initial depletion and subsequent recovery observed in experimental animals also occurs in man. There are some important differences, however.

Radiation-induced depletion of the spermatogonial population of the mouse is primarily the result of cell killing [38], whereas both cell killing and differentiation into more mature cell types appear to be important in man. Cell killing results in the initial rapid decrease in the spermatogonial population in man, but the gradual decline which continues until 90 days or more is the result of both cell death and spermatogonial differentiation.

As demonstrated by a comparison of Figs. 1 and 2, repopulation of the seminiferous epithelium also is much slower in man than in the mouse. Comparable data for type A spermatogonia after 300 R in the mouse also are included in Fig. 2 for ease of comparison. The difference is greater than would be expected on the basis of duration of spermatogenesis, for the time required in man is only 72 days compared with 35 days in the mouse. Obviously, other factors are responsible,

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and these may concern basic processes in the control of spermatogonial differentiation. In the mouse, the A spermatogonia first increase in number, then differentiate into intermediate and B spermatogonia. In man, however, differentiation into B spermatogonia occurs when the number of Ad cells still is as low as 2 or 3% of control. This represents a "wastage" of cells as far as recovery of the Ad population is concerned, and appears to be a significant factor in the slow repopulation of the radiation depleted human testis.

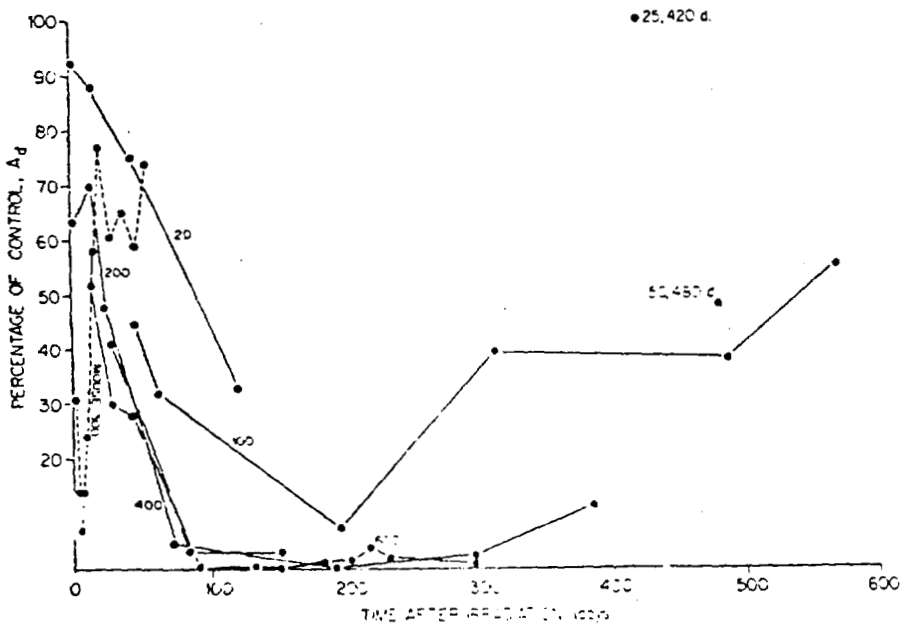


FIG. 2. Experimental/control ratios for total Ad spermatogonia of man after X-ray exposure.

Comparison of the doses required for comparable depression of spermatogonia in mouse and man suggests that man is the more sensitive by a factor of about 3. However, this estimate must be accepted with cogent reservations. First, the comparison is based on data at 5-7 days after irradiation in the mouse, and on data at 90-200 days in man. Second, the mechanisms of spermatogonial depletion differ in mouse and man. Third, the dynamics of re-establishment of the spermatogonial population in man reveals significant differences in the control of spermatogonial differentiation in mouse and man. Thus, while the general radiation response of the human testis is typical of all mammals, many aspects of the normal process of gametogenesis will have to be more thoroughly understood before radiation sensitivity of experimental animals and man can be adequately compared.

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## DISCUSSION

F.H. SOBELS: You mentioned that the period of high genetic radiosensitivity at the end of the dictyate stage of the mouse oocyte seems to coincide with cessation of RNA metabolism. Do you or Dr. Russell know of any other evidence that the apparent repair of premutational damage at earlier stages is associated with RNA metabolism?

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E. F. OAKBERG: At present we can only say that RNA metabolism is an indicator of general metabolic activity, which may be connected with repair systems.

L. B. RUSSELL: The low mutation frequency obtained when early follicle stages are sampled may be due to mechanisms other than repair, such as cell selection.

E. F. OAKBERG: A third possibility is that it is connected with a possible difference in intrinsic sensitivity of the genetic material, as W. L. Russell has pointed out (Proc. natn. Acad. Sci. U. S. A. 54 (1965) 1552 - 57).

F. H. SOBELS: What is the mechanism of cell death in the B type spermatogonia?

E. F. OAKBERG: The precise mechanism is unknown, but there is an immediate cell killing which is already detectable 3 - 4 hours after the irradiation in both mouse and man. In man, many of them differentiate into spermatocytes, which is a different response from what we see in the mouse or rat. We really do not understand how the type A spermatogonial population maintains itself. Recently Clermont and his group have suggested there is a so-called $A_0$ spermatogonium which is a true stem cell forming a reserve population to replenish the seminiferous epithelium. One interpretation of the human results could be that the actual spermatogonial differentiation in man occurs more than 72 days before production of mature sperm, but that we are unable to recognize the initial cell population, so we have the wrong estimate of the duration of human spermatogenesis.

H. M. BEAUMONT: I wonder if you would comment on cell selection in oocytes.

E. F. OAKBERG: We certainly have evidence for increased cell selection through atresia in the irradiated female. It is interesting that the female can ovulate about the normal number of oocytes each oestrus even though she has very few left. But atresia occurs at different times in the control and irradiated females. In the irradiated female degeneration occurs at oocyte stages 1 - 5; however, once a follicle has developed an antrum the oocyte has a better chance of being ovulated in the irradiated than in the control. The increased loss in the irradiated females must be due to selection against those oocytes bearing the more severe types of damage, probably chromosomal.

A. G. SEARLE: Is it known how much the duration of oocyte stages 6 - 8 depends on whether the female is having litters or not?

E. F. OAKBERG: The data on this are not good, because we cannot follow a specific oocyte. There is no difference in the rate of oocyte loss between virgin and continuously mated females; the depletion of the oocyte pools occurred at exactly the same rates. This may result from the fact that most of the loss of oocytes comes through atresia rather than through ovulation.

L. B. RUSSELL: Another result relevant to this point comes from our experiments with 50 R acute X-irradiation given to female mice. When the young were killed at birth, as done in the fertility experiment, an average of four litters was produced. But when you allow them to be raised, as done in the mutation-rate experiments, the average number of litters is smaller because they are more widely spaced. This also points to loss through atresia rather than through ovulation.

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A. G. SEARLE: You gave a figure of 46 days for the interval between rmation of the primary spermatocyte and of the spermatozoon in man. hat is the minimum time between spermatozoal formation and ejaculation?

E. F. OAKBERG: The minimum time between release of the permatozoa in the tubule and their ejaculation is one day, but the average s probably 10 - 14 days.

A. J. BATEMAN: Is there any evidence for premature death of the permatocyte before meiotic division?

E. F. OAKBERG: There is no evidence of any loss at this stage. Normal numbers of spermatocytes reach division even with very high exposure, apart perhaps from some at the very early preleptotene stage. Most of the degeneration occurs at metaphase I, so the scoring of abnormal maphases may be misleading.

A. J. BATEMAN: An irradiated spermatocyte normally forms what is called a restitution cell. Were they scored as survivors or non-survivors?

E. F. OAKBERG: In my opinion a restitution cell is a figment of the imagination. The concept of a restitution cell is erroneous because the spermatocytes which fail in meiosis form a giant spermatid which degenerates. These cells play no role whatsoever in repopulation of the testis. Investigators who have made this claim simply did not look long enough to find the few A type spermatogonia present.

We scored as survivors all those spermatids which appeared to be normal living cells, regardless of nuclear size.

A. J. BATEMAN: If you give a larger dose of about 1000 rads you get very many spermatocytes which have failed to go through meiosis. They a ulate in the testes and as a cell population can legitimately be called r tion cells. 'Restitution' is the classical cytological term for process by which two daughter nuclei fuse, failing to separate, are included within a single nuclear membrane.

E. F. OAKBERG: That is true, but these cells have nothing to do with testes repopulation and the tem 'restitution cell' implies that they do.

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TABLE 1

COMPARISON OF THE VARIABILITY IN QUANTIFICATION OF THE SAME TESTIS IN THE SAME SUBJECT AT DIFFERENT TIMES

| Subject no. | Date of biopsy | Testis side | Leydig cell/Sertoli cell ratio ± S.E. | P* |
|---|---|---|---|---|
| 50 | 3 8 67 | Right | 805/3050 = 0 26 ± 0 03 | N S |
| | 21 9 67 | Right | 670/2448 = 0 27 ± 0 03 | |
| 44 | 16 6 66 | Right | 1956/3698 = 0 53 ± 0 03 | N S |
| | 3 8 67 | Right | 1374/2516 = 0 55 ± 0 03 | |
| 180 | 10 1 63 | Right | 2049/3996 = 0 51 ± 0 03 | N S |
| | 21 9 67 | Right | 1681/3229 = 0 52 ± 0 04 | |
| 206 | 26 1 67 | Right | 1102/3216 = 0 34 ± 0 03 | N S |
| | 3 8 67 | Right | 850/3081 = 0 28 ± 0 03 | |
| | 21 9 67 | Right | 1415/4141 = 0 34 ± 0 03 | |
| 206 | 26 1 67 | Left | 1084/3064 = 0 35 ± 0 03 | N S |
| | 3 8 67 | Left | 1165/3297 = 0 35 ± 0 03 | |
| | 21 9 67 | Left | 1364/3687 = 0 37 ± 0 03 | |

N S , Not significant

* For this report, $P < 0.05$ is the criterion for statistical significance

TABLE 2

COMPARISON OF THE VARIABILITY BETWEEN SUBJECTS

| Subject no. | Testis side | Leydig cell/Sertoli cell ratio ± S.E. |
|---|---|---|
| 227 | Right | 2700/5934 = 0 46 ± 0 03 |
| | Left | 3083/4510 = 0 68 ± 0 03 |
| 180 | Right | 2049/3996 = 0 51 ± 0 03 |
| | Right | 1681/3229 = 0 52 ± 0 01 |
| 262 | Right | 876/4277 = 0 20 ± 0 01 |
| | Left | 1286/5896 = 0 22 ± 0 01 |
| 224 | Left | 2720/5660 = 0 39 ± 0 02 |
| 223 | Right | 2955/5521 = 0·54 ± 0 03 |
| | Left | 2794/7995 = 0 35 ± 0 02 |
| 189 | Right | 3534/6162 = 0 57 ± 0 01 |
| | Left | 5843/6769 = 0 57 ± 0 02 |
| 204 | Right | 2994/8016 = 0 37 ± 0·02 |
| 50 | Right | 670/2448 = 0 27 ± 0·03 |
| | Right | 805/3050 = 0 26 ± 0 03 |
| | Left | 769/2634 = 0 29 ± 0 02 |
| 219 | Left | 1074/3323 = 0 32 ± 0 03 |
| 218 | Right | 1056/3956 = 0 27 ± 0 01 |
| | Left | 1111/5845 = 0·19 ± 0·01 |
| 170 | Right | 1882/8996 = 0 21 ± 0 01 |
| 232 | Right | 3173/8507 = 0 37 ± 0 01 |
| 260 | Right | 1848/6751 = 0 27 ± 0 01 |
| | Left | 1122/4410 = 0 25 ± 0·02 |
| 44 | Right | 1956/3698 = 0 53 ± 0 03 |
| | Right | 1374/2516 = 0 55 ± 0·03 |
| | Left | 5076/4614 = 0·67 ± 0·03 |
| 206 | Right | 1102/3216 = 0 34 ± 0·03 |
| | Right | 850/3081 = 0·28 ± 0·03 |
| | Right | 1415/4141 = 0 34 ± 0 03 |
| | Left | 1165/3297 = 0 35 ± 0 03 |
| | Left | 1084/3064 = 0·35 ± 0 03 |
| | Left | 1364/3687 = 0 37 ± 0 03 |
| 199 | Right | 1528/3365 = 0·45 ± 0 03 |
| | Left | 2852/6109 = 0·47 ± 0·02 |
| 251 | Left | 5067/7037 = 0·72 ± 0·04 |
| 243 | Right | 3059/7117 = 0·43 ± 0·02 |
| | Left | 4169/8683 = 0·48 ± 0·02 |
| 150 | Left | 2064/9227 = 0 22 ± 0 01 |



(c) *Comparison of biopsies from the right and left testes of the same subject*

The comparison of LC/SC ratios between right and left testis of the same subject obtained at the same time revealed a significant difference in four of the eleven men. The ratios of the right and left testis did not vary significantly in seven men ($P = 0.05$) (Table 3). Statistical analysis showed that the variation between subjects was greater than between right and left testes of the same

TABLE 3

COMPARISON OF THE VARIABILITY OF THE TWO TESTES ON THE SAME SUBJECT AT THE SAME TIME

| Subject no. | Right testis Leydig cell/Sertoli cell ratio ± S.E. | Left testis Leydig cell/Sertoli cell ratio ± S.E. | P* |
|---|---|---|---|
| 227 | 2700/5934 = 0 46 ± 0 03 | 3083/4510 = 0 68 ± 0 03 | < 0 005 |
| 262 | 876/4277 = 0 20 ± 0 01 | 1286/5896 = 0 22 ± 0 01 | N S |
| 223 | 2955/5521 = 0 54 ± 0 03 | 2794/7995 = 0 35 ± 0 02 | < 0 005 |
| 189 | 3534/6162 = 0 57 ± 0 01 | 5843/6769 = 0 57 ± 0 02 | N S |
| 50 | 805/3050 = 0 26 ± 0 03 | 769/2634 = 0 29 ± 0 02 | N S |
| 218 | 1056/3956 = 0 27 ± 0 01 | 1111/5845 = 0 19 ± 0 01 | < 0 005 |
| 260 | 1848/6751 = 0 27 ± 0 01 | 1122/4410 = 0 25 ± 0 02 | N S |
| 206 | 1102/3216 = 0 34 ± 0 03 | 1165/3297 = 0 35 ± 0 03 | N S |
| | 850/3081 = 0 28 ± 0 03 | 1084/3064 = 0 35 ± 0 03 | |
| | 1415/4141 = 0 34 ± 0 03 | 1364/3687 = 0 37 ± 0 03 | |
| 44 | 1956/3698 = 0 53 ± 0 03 | 5076/4614 = 0 67 ± 0 03 | < 0 025 |
| 199 | 1528/3365 = 0 45 ± 0 03 | 2852/6109 = 0 47 ± 0 02 | N S |
| 243 | 3059/7117 = 0 43 ± 0 02 | 4169/8683 = 0 48 ± 0 02 | N S |

N S , Not significant

* For this report, $P < 0.05$ is the criterion for statistical significance

subject. Reproducibility was revealed by re-counting the same biopsy by the same person and by different persons. The variation in counts performed by any two investigators averaged less than 6% and the variation in counts by the same investigator was within 3%.

## DISCUSSION

The LC/SC ratios reveal that the number of Leydig cells remains remarkably constant for the same testis in a given subject over a period of time. Hence, analysing biopsies of the same testis in the same individual before and following an investigative procedure should reveal changes with a validity of $P = 0.05$. Conversely, since the two testes of the same individual have approximately a 40% chance of being statistically dissimilar, comparing the right testis used as a control with the left testis after treatment could be misleading.

Similarly, using the average LC/SC ratio of a group of normal controls for assessing the results of a given procedure upon an individual or a small group of individuals could be even more misleading. Therefore, each subject must serve as his own control and each testis must also serve as its own control.

To date, no correlation has been forthcoming to rationalize the great

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differences in LC/SC ratios between different subjects. Age was found not to be a factor as no significant correlation ($r = -0.23$) was found between age and the LC/SC ratios of the subjects. Other possibilities to be considered are that indeed, some individuals may have more Leydig cells than others, or, conversely, some may have more Sertoli cells than others. Although the total number of Sertoli cells per subject may vary, further stability of the Sertoli cell was demonstrated by counting the number of Sertoli cells per tubular cross section. The average number of Sertoli cells per tubule for all subjects studied was $10.03 \pm 0.3$. Table 4 reveals that the number of Sertoli cells/tubular cross section for the two subjects whose LC/SC ratios lay at the extreme ends of the range averaged $10.36 \pm 0.6$, well within the average range found for all subjects.

TABLE 4

COMPARISON BETWEEN SERTOLI CELLS/TUBULAR CROSS SECTION FOR TWO SUBJECTS WHOSE LEYDIG CELL/SERTOLI CELL RATIOS LAY AT EXTREME ENDS OF THE RANGE

| Subject no. | Testis side | Leydig cell/Sertoli cell ratio ± S.E. | Sertoli cell/tubular cross section ± S.E. |
|---|---|---|---|
| 218 | Right | 0.27 ± 0.01 | 879.83 = 10.59 ± 0.42 |
| | Left | 0.19 ± 0.01 | 1001.90 = 11.16 ± 0.47 |
| 251 | Right | 0.75 ± 0.04 | 676.90 = 8.5 ± 0.38 |
| | Left | 0.72 ± 0.04 | 891.80 = 11.18 ± 0.48 |

It is conceivable that a higher testosterone production may be correlated with either a change in Leydig-cell numbers or a change in Leydig-cell size. The increased testosterone production with human chorionic gonadotrophin is correlated with an increase in their size (Heller & Leach, 1971), whereas following irradiation, preliminary analysis in our laboratory reveals that subsequent diminution in testosterone secretion may be correlated to an increase in Leydig cell numbers, thereby suggesting a compensatory mechanism.

Other methods for quantifying Leydig cells were unsatisfactory because control biopsies could not be compared with biopsies following administration of drugs and hormones. The method of Chalkley (1943) determines the volume of Leydig cells present in a sample, but does not reveal numbers.

Sargent & McDonald (1948) related the number of Leydig cells to the number of tubules present on any given area of the biopsy. However, this did not provide for changes in the size of the tubules following administration of various agents. Clegg (1961), using rats, modified Sargent & McDonald's method by deriving a mathematical formula to compensate for shrinkage of the tubules. A uniform distribution of the cells in the testes was assumed along with a similar shrinkage of the tubules with regard to diameter and length. Since there is no evidence that a similar ratio of shrinkage between diameter and length of tubules occurs in man, we could not use this method.

The method of Dykes (1969) measures the relative proportions of tubules, areas of Leydig cells in normal and pathological testes. However, this point counting method makes no mention of Leydig-cell numbers, only of area or volume, and does not take into account the possible changes in tubular



which may occur following administration of various agents to the normal subject. Ahmad, Lennox & Mack (1969) tried to improve on Dykes' method by measuring the total volume of the testes and the proportion of the testes occupied by the cells. This method could not be used because it also makes no mention of Leydig-cell numbers. The authors did not have an adequate series of normal controls since the testicular material was obtained *post mortem* and no definite conclusions were reached because of the small number of cases studied.

The fact that the Sertoli cells are located in the tubules and the Leydig cells in the interstitial area does not enter into the statistical analysis. This is because the tubules are not found in one area of the testes and the interstitial tissue in another. Since the tubules and Leydig cells are homogeneously mixed throughout the entire testes, the LC/SC ratio is assumed to be a normally distributed random variable. In species where the tubules and interstitial tissue are in separate discrete areas, this assumption is not valid and, therefore, the method cannot be used to quantify Leydig cells.

The biopsy specimens are obtained from a limited area of the dorsal side of the testes immediately below the tunica albuginea to avoid any disturbance of the epididymis. Only a small area of the testis is sampled, not the entire organ, so the homogeneity of the biopsy specimens and of the association of cells to tubules is stable because the area of the testis sampled is always the same.

The LC/SC ratio is valid as long as a statistically random sample is scored. In testing the method, the authors have counted an excess of twenty sample areas of the biopsy specimen. The investigator may quantify a larger number of sample areas of the biopsy to obtain the LC/SC ratio, though it is only necessary to count a minimum of ten and statistically evaluate the data as set forth in the method. If the data are not statistically valid, more areas may be counted, though the LC/SC ratio from counting ten sample areas is usually within 10% of the true ratio ($P < 0.90$).

When dealing with human tissue, one is limited to the use of biopsies, except in some pathological cases. Because it is impossible to weight the entire organ and study it as such, this method enables the investigator to obtain information concerning the relative number of Leydig and Sertoli cells in any individual man. Since it utilizes the least amount of tissue with the least harm to the individual, the investigator will be able to determine the effects of any investigational procedure on the same individual by means of multiple biopsies over a period of time.

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# QUANTIFICATION OF LEYDIG CELLS AND MEASUREMENT OF LEYDIG-CELL SIZE FOLLOWING ADMINISTRATION OF HUMAN CHORIONIC GONADOTROPHIN TO NORMAL MEN

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**Summary.** Six normal men were injected with 4000 i.u. of HCG intramuscularly three times a week for 6 weeks or every 2nd day for 16 weeks. Levels of urinary testosterone and oestrogens were measured. Leydig cells were quantified on the basis of the Leydig cell/Sertoli cell ratio, and Leydig-cell size was measured.

Following both 6 and 16 weeks HCG administration, four subjects showed no change in Leydig-cell numbers. The remaining subjects showed slight increases or decreases. Levels of urinary testosterone and oestrogen increased. It is concluded that there is no consistent increase in Leydig-cell numbers following HCG administration. Measurements of Leydig-cell nucleus and cytoplasm revealed a significant increase in the size of both at 6 and 16 weeks in all but one subject. It is concluded that the increased activity of the Leydig cells is related to their increase in size and not to an increase in number as had been reported.

## INTRODUCTION

The Leydig cells of the normal adult human male are stimulated by interstitial-cell-stimulating hormone (ICSH) to produce androgens and oestrogens. Their normal function may be altered by the administration of certain drugs and hormones. Among these are testosterone which depresses their activity and human chorionic gonadotrophin (HCG) which stimulates their activity.

Stimulation of Leydig cells by HCG is reflected by increased production of 17-ketosteroids (Maddock & Nelson, 1952; Maddock, Epstein & Nelson, 1952; Leach, Maddock, Tokuyama, Paulsen & Nelson, 1956; Tamm, Apostolakis & Voight, 1965), oestrogen (Maddock & Nelson, 1952; Maddock *et al.*, 1952; Leach *et al.*, 1956; Tamm *et al.*, 1965) and testosterone (Tamm *et al.*, 1965; Cleveland, Ahmad, Sandberg & Savard, 1966). It was thought this increase could be correlated with cytological observations. Indeed, Maddock & Nelson (1952) reported that the increase in the activity of the Leydig cells was correlated with an increase in their numbers. However, Maddock & Nelson (1952)



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