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TESTICULAR FUNCTION AND THE EFFECTS OF MICROWAVE RADIATION

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NOTICES


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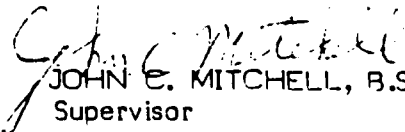
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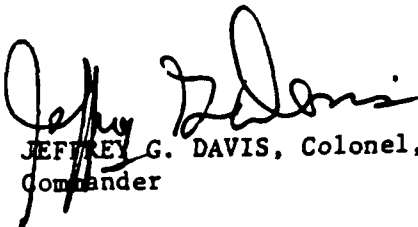
The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources-National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.


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TESTICULAR FUNCTION AND THE EFFECTS OF MICROWAVE RADIATION

OVERVIEW AND RATIONALE

We carried out a series of experiments to quantitatively characterize the effects of microwave radiation (MWR) on sperm production and testicular function in the adult male rat. In the Sprague-Dawley strain, the cycle period of the seminiferous epithelium is 13 days. One full cycle of spermatogenesis comprises four such elemental cycles, for a total of 52 days. So as to be able to sample physiologic and morphologic variables relating to sperm production at the several stages of spermiogenesis, our basic protocol called for treatment then sacrifice of the animals for analysis at 6.5, 13, 26 or 52 days post-treatment. Conventional thermal and MWR induced effects were examined using this same basic protocol. Preliminary studies had indicated that in the freely mobile rat, 9 days of exposure at 6.3 mW/g (6 h/day) did not yield significant changes in testicular parameters despite evident whole-body thermogenesis. We proposed that this could be due, in part, to the noncumulative effect of MWR at this dose rate on spermatogenesis. Since secondary and not necessarily relevant behavioral thermoregulatory mechanisms could cause gross underestimation of maximal potential testicular effects, a more biophysical approach to the problem was proposed.

In the first series of experiments carried out under this contract, male Sprague-Dawley rats were exposed to MWR at an acute thermogenic dose rate (9 mW/g) for 8 h. MWR exposure and otherwise comparable sham-irradiation treatment took place in unidirectionally energized cylindrical waveguide sections within which the animals were essentially unrestrained. Such exposure was found to yield an elevation of deep rectal temperature to 40.5°C. Net mass of testes, epididymides and seminal vesicles, daily sperm production (DSP) per testis and per gram of testis, sperm morphology, and the number of epididymal sperm were determined. The levels of circulating follicle stimulating hormone (FSH) and leutinizing hormone (LH) were derived via radioimmunoassay of plasma samples taken at the time of sacrifice. Despite the evident thermogenic level of the MWR dose rate, no decrement in testicular function was found.

These preliminary studies showed that, in the unrestrained rat, whole-body hyperthermia was not a sufficient condition for disruption of any of the key measures of testicular function. We concluded that the conventional approach, namely, environmental exposure of the alert and mobile animal, was unsuited to a quantitative description of the effects of MWR on testicular function. More precise control over the regional (testicular) MWR dose rate needed to be explored as an initial strategy. For this an anesthetically immobilized preparation was devised, in which the relationship between hindquarter orientation and incident field could be kept within a limited range of variation.

The effects of acute exposure to pulse-modulated (PM) MWR at 1.3 GHz on testicular function in the immobilized rat were studied next. Anesthetized adult males rats were treated then sacrificed at specific intervals with

respect to the 13-day cycle of the seminiferous epithelium, as described above. Positive thermal control procedures included the measurement, in similarly anesthetized animals, of the changes in rectal and testicular temperatures during warm-water immersion of the testicles. The changes in potential DSP and allied spermatogenic variables subsequent to either MWR-induced or conventional heating were subsequently compared. The time course of rectal and testicular temperatures during irradiation was likewise determined for each exposure condition.

PM MWR at 7.7 mW/g for 90 min was adequate for the production of a modest decline in DSP that, because of its appearance at 26 days post-treatment, was correlated with a maximum sensitivity of germ cells at the spermatocyte stage. PM MWR at 4.2 mW/g for 90 min was ineffective with respect to all of the measured parameters. The intratesticular temperature achieved during the former was on the order of 40°C whereas with exposure at 4.2 mW/g testicular temperature did not exceed 38°C. Exposure at a MWR dose rate considerably in excess of 7.7 mW/g produced germ cell damage at virtually all stages of maturation but again with the apparent maximal effect seen 26 days after treatment. Using conventional heating, a rise in intratesticular temperature to a level in excess of 39°C was required to produce significant physiologic decrement in DSP, in agreement with the established literature on conventional heat effects. We concluded that the damage threshold and the differential sensitivity of immature germ cells to PM MWR could be adequately explained by the macroscopic heating produced thereby.

In a supportive series of studies we compared the decrements in testicular function of acute exposure to PM versus continuous-wave (CW) MWR. Both CW and PM MWR at 7.5 mW/g for 90 min were adequate for the production of a modest decline in the number of epididymal sperm. This decline was most evident at 26 days post treatment; other measures of testicular function (daily sperm production, total testes mass) yielded a consistent profile. The appearance of the most significant changes at 26 days post treatment again suggested a maximum sensitivity of germ cells at the spermatocyte stage, with CW or PM MWR being equally effective. The possibility of a difference in susceptibility at other stages of maturation was raised, however. The time course of rectal and testicular temperatures during irradiation (using weight-matched surrogate animals) indicated that both PM and CW exposures at 7.5 mW/g had similar effects on testicular temperature. We concluded that, since the damage threshold and the differential sensitivity of immature germ cells to PM and CW MWR are quantitatively the same, average and not peak power density would appear to be the critical factor.

While the work schedule did not permit all aspects of our more biophysical approach to be carried out (a complete description of testicular SAR remains to be done), the studies reported here constitute a novel and quite productive approach to the study of the impact of MWR on mature and developing mammalian male germ cells. The methodology is clearly capable of providing the quantitative data necessary for definition of the much needed three-way correlation among dose variables (testicular dose rate, whole-body dose rate and testicular temperature rise) versus the differential sensitivity to MWR of germ cells at different stages of maturation. On the basis of the work completed to date, we can discern no contraindications to the view that macroscopic heating is the most direct explanation for the results obtained at this single frequency of irradiation.

INTRODUCTION

At very high dose rates, and consistent with the thermal sensitivity of testes (1), evident testicular degeneration (loss of spermatozoa, fusion of spermatid nuclei) has been reported to result from exposure to microwave radiation (2). Additional detailed testicular cytological studies that have enhanced our ability to define reliable hazard thresholds have appeared (3,4,5); verification and extension of such studies are evident needs.

Our major concern with work published to date has been the substantial reliance upon data from unrestrained, freely mobile animals and a lack of direct evidence of the extent of specific testicular absorbed dose. Some control over the MWR dose deposited within the relevant target organ, here the testis, would seem to be a minimal requirement for any dose/response analysis.

Testes have an exocrine function (sperm production) as well as endocrine function (production of male hormone, testosterone). Both functions should be considered when evaluating the impact of potential hazards. The production of testosterone can be measured from blood samples via radioimmunoassay or estimated in bioassay through measurement of epithelial height or weight of the seminal vesicles (6). For evaluation of the exocrine function of the testes, sperm production rates expressed as DSP (7,8), number of epididymal sperm, and morphology of epididymal sperm can be evaluated. While DSP/testis is a measure of the total number of sperm produced, DSP/g is a measure of the efficiency of sperm production by the seminiferous epithelium. The number of stored epididymal sperm in celibate males is influenced by the level of sperm production; hence, the number of epididymal sperm corroborates observed changes in rates of sperm production.

Evaluation of testicular histology or of reproductive efficiency in breeding trials is sufficient to detect large changes in spermatogenesis. However, more directly quantitative expressions of spermatogenesis such as the DSP are needed to detect less pronounced changes (9). Larry Johnson (Co-investigator) has employed such an assay system to evaluate spermatogenesis in various species including rats and humans (10,11,12). Good agreement has been found between the DSP determined from human testes (12) and the daily sperm output determined from the number of sperm in human ejaculates (13).

In previous reports we indicated that repeated daily exposure to MWR at 1.3 GHz, at a dose rate of 6.3 mW/g, 6 h/day for 9 days did not significantly perturb testicular function in the adult male rat (14,15). At no stage in the development of the male germ cells could a significant physiologic or morphologic deficit be found. The relative lack of responsiveness of testicular physiologic and morphologic variables subsequent to acutely thermogenic levels of MWR has been noted by others (3,5). To induce a deficit in testicular function, dose rates that exceed the thermal accommodation capacity of the test animal appeared to be required (2,16). This result is somewhat puzzling in view of the evident sensitivity of testis to heating (17). A resolution exists, perhaps, in the manner in which the independent variable (level of MWR exposure) is quantified with respect to the target organ.

In order to minimize the confounding factors and to better define testicular sensitivity to MWR, we developed an anesthetically immobilized preparation for these studies. We thus were able to stabilize the regional (testicular) dose rate and induce known, incremental elevations of testicular temperature. Our approach was to examine testicular function at discrete intervals following the irradiation, which intervals corresponded to multiples of the 13-day cycle of the seminiferous epithelium of the Sprague-Dawley rat (18). The objective was to determine the effects of MWR on testicular function measured at the time periods following exposure corresponding to 0.5, 1, 2, and 4 cycles of the seminiferous epithelium in rats (6.5, 13, 26 and 52 days post-treatment, respectively). The discrete sampling intervals procedure allowed us to determine which cell type (spermatogonia, spermatocyte, or spermatid) in spermatogenesis was most sensitive to microwave treatment. To directly link these data to the well developed literature on the thermal sensitivity of the testis, companion experiments utilizing water immersion of the testis were carried out.

METHODS

Approximately 500 adult Sprague-Dawley male rats between 400-500 g were used for these studies. These were housed on a 12-12 h light-dark cycle and all irradiation, thermal treatment, and thermal profile studies were carried out in the morning (light on). Animals were encoded by ear-punch and all post-treatment procedures were carried out in the blind.

For the irradiation studies, groups of 32 rats were randomly assigned to either an irradiation or sham-irradiation subgroups (n=16). Occasional rats had to be deleted if they did not survive throughout the required post-treatment interval or if they demonstrated unrelated acute ill health. MWR exposure took place in individual cylindrical waveguides operating in a circularly polarized, unidirectional mode (19). Animals of the sham-irradiation (control) subgroups were treated in a fashion identical to that of the irradiated animals but placed in unenergized waveguide chambers. Specific absorbed-dose rates (SAR) for the irradiated subgroups were specified by group mean SAR. Groups of 32 animals were run in parallel at controlled ambient temperature (23 ± 1.5 °C) and relative humidity (mean 37%, range 32 to 45%). In separate experiments, irradiated subgroups were exposed to MWR at 1.3 GHz either CW or PM (1 μ sec pulse width, 600 pulses/sec). When not in the waveguides, the animals were group housed (random grouping, with food and water available, ad lib).

Unrestrained, freely mobile animals were used in our initial studies. The majority of the experiments, however, used an anesthetically immobilized preparation. Animals were anesthetized with sodium pentobarbital (55 mg/kg IP), a dose sufficient to insure that the animal remained in position throughout a 90-min exposure period. The animals were placed within the actual or sham-irradiation waveguide when immobile and periodically viewed; if the animal recovered from this anesthetic dose and moved out of position during exposure, the animal was deleted from further study.

The time course of deep rectal and testicular core temperatures was determined in anesthetized, surrogate animals for each MWR exposure condition, using MWR transparent temperature probes (Vitek). Since both testes were required of each animal for physiological analysis and since monitoring intratesticular temperature required a physiologically damaging needle puncture, temperature and physiological data were derived from different groups of weight matched animals.

Positive control studies were carried out to characterize the thermal consequences and subsequent changes in testicular function following immersion of the testicles in temperature-regulated water baths. Using the procedures of Setchell and Waites (20), anesthetized animals were suspended at a 45° angle with the testicles fully immersed for 60 min in water maintained at 34, 39, 41 or 43°C. The tail and hind limbs were kept out of the water as much as possible. Again, since monitoring intratesticular temperature required a physiologically damaging needle puncture, temperature and physiological data were derived from different groups of weight matched animals.

The animals were taken for study at either 6.5, 13, 26, or 52 days following the treatment day, corresponding to 0.5, 1.0, 2.0, and 4.0 cycles of the seminiferous epithelium of the Sprague-Dawley rat (21,22). On the date of termination, each rat was weighed then decapitated, a sample of trunk blood being taken for subsequent neuroendocrine assay. The testes were separated from epididymides before the weights of each testis, epididymis, and seminal vesicles were noted. The right testis was decapsulated (noting weight of the tunica albuginea) and homogenized for 2 min in 100 ml of homogenizing fluid containing 150 mM NaCl, 0.05% (v/v) Triton X-100, and 3.8 nM NaN₃ (11). Testicular homogenates were stored at 5 °C and evaluated within 28 h. Only spermatid heads with a shape characteristic of steps 17 through 19 found in stages IV and VIII (22) are resistant to such homogenization; these were enumerated by phase-contrast cytometry. DSP/testis was calculated by dividing the number of homogenization-resistant spermatids by the life span of 6.3 days (10¹¹). DSP/g parenchyma (DSP/g) was calculated by dividing the DSP/testis by the difference between testis and tunic weights. The number of sperm in the right epididymis was determined from epididymal homogenates prepared and evaluated as for the testes.

The left testis from each rat was fixed immediately by vascular perfusion with 2% glutaraldehyde in 0.1-M sodium cacodylate buffer at pH 7.4, osmicated, sectioned at 0.5 μm, stained with toluidine blue and observed under bright-field optics (11). For qualitative evaluation of spermatogenesis, the tail of the left epididymis was diced in 2 ml of Minimum Essential Medium (MEM, Grand Island Biological) at room temperature and sperm allowed to swim from the tissue into the free medium. A smear of sperm was placed on each of two glass slides and stained with nigrosin and eosin. At least 100 sperm from each slide were classified as either normal or abnormal under bright field microscopy, using the criteria of Wyrobek (23).

Circulating levels of LH and FSH were determined from the trunk blood sample using established radioimmunoassay techniques (24).

Statistical validation of significant treatment effects was accomplished by a two-way (treatment by post-treatment sample interval) analysis of variance (ANOVA), correcting for unequal cell numbers using the unweighted means procedures of Winer (25). Simple t-tests or a studentized

Newman-Keuls (SNK) multiple means range test was subsequently applied, as appropriate, to uncover individually significant differences between group means.

RESULTS

Eight-hour Exposure of the Mobile Rat

Preliminary experiments (14,15) had shown that prolonged exposure to high levels of MWR would not produce significant decrements in testicular functioning. These data, and associated thermal control studies, suggested that it would be inappropriate to derive safety standards from such a protocol. Prolonged exposure mimics the workplace or environmental situation but are a weak basis for studying the causal chain in MWR-induced decrements in testicular function. Further, the preliminary studies made clear the need to derive data that could be unambiguously related to the potential differential sensitivity of germ cells at their successive stages of maturation. Irradiation over a period of several days does not allow such an unambiguous assignment of cellular changes to the spermatogenic cycle. We proceeded, therefore, to a more acute exposure protocol.

A whole-body exposure for 8 h at a significantly thermogenic SAR was used (1.3 GHz CW at 9 mW/g). A summary of key physiological data, for all treatment groups, is presented in Table 1. The calculated DSP (mass normalized to DSP/g parenchyma) in terms of cycle periods of the seminiferous epithelium, are shown in Figure 1 for a total of 30 rats. A two-way ANOVA revealed no significant treatment effect ($F(1,22)=.090$; $p=.764$). Potential DSP calculated on a per testis basis was similarly unaffected by the exposure ($F(1,22)=1.059$; $p=.316$). The number of epididymal sperm did appear to be reduced at 26 days following treatment (Fig. 2). However, ANOVA indicated that no overall treatment effect could be substantiated ($F(1,22)=.104$; $p=.748$). There was only a marginal indication of interaction between irradiation and the postirradiation sample period ($F(3,22)=2.676$; $p=.0713$). The group mean at 26 days following treatment was, in fact, significantly lower ($p<.05$) for the irradiated animals; however, a simple a posteriori t-test is not rigorous in view of the negative ANOVA and in isolation from the other sample means. Since confirmation of such a weak interaction was not found in other aspects of these studies (presented below), we cannot rule out that this was a purely aberrant result.

Bioassay of testosterone levels via seminal vesicle weight (cf. Table 1) showed no apparent significant differences due to irradiation, although there was a single instance of a significant difference in subgroup means, again at the 26-day posttreatment latency. This measure reflected the response to collective circulating testosterone over a period of time. However, if a reduction in testosterone levels were a result of irradiation, one would expect to observe a relative increase rather than the decrease in seminal vesicle size that was marginally suggested by these data. Two-way ANOVA did not support the conclusion that there were any significant treatment dependent main ($F(1,22)=2.178$; $p=.151$) or interaction ($F(3,22)=.638$; $p=.602$) effects. More to the point, direct measurement of circulating FSH and LH in the trunk blood taken at the time of termination (Fig. 3) did not show any effect of

TABLE 1 . PHYSIOLOGICAL PARAMETERS FOLLOWING CW MWR AT 9 MW/G

VARIABLE / mean se	SPERMATOGENIC CYCLE							
	0.5		1.0		2.0		4.0	
	SHAM	IRRAD	SHAM	IRRAD	SHAM	IRRAD	SHAM	IRRAD
(N =	4	3	4	3	4	4	4	4)
Body mass (g)	487.4 4.3	479.0 12.9	511.8 4.2	498.9 13.0	519.9 12.1	512.4 12.0	563.5 7.5	552.1 19.1
Tot. testis ms. (g)	3.01 .03	3.20 .17	3.13 .14	3.09 .02	3.33 .14	3.24 .16	3.06 .12	3.38 .17
Rt. tunic (mg)	70.7 4.9	72.8 8.2	61.8 7.7	55.2 4.7	69.7 3.7	74.6 4.4	75.8 3.4	80.5 7.3
Lft. epidid. (g)	.534 .019	.543 .026	.523 .009	.400 .127	.582 .037	.537 .018	.557 .014	.566 .020
DSP/testis (x 10 ⁶)	31.0 1.0	37.0 3.5	34.1 2.9	31.7 2.0	31.5 0.7	31.1 2.4	34.7 2.2	37.9 1.9
DSP/gam (x 10 ⁶)	22.1 0.6	24.5 1.3	22.9 1.5	21.8 1.2	20.4 0.5	20.6 0.5	24.5 0.6	23.8 0.2
Rt. epid. sperm count (x 10 ⁶)	250.5 19.7	267.0 36.3	265.7 24.8	281.2 13.0	311.2 16.5	234.8* 19.7	283.2 20.1	308.6 10.3
seminal vesic. (g)	1.66 .11	1.69 .20	1.74 .15	1.75 .06	1.53 .08	1.83* .05	1.56 .04	1.73 .18

* - Difference between means significant (t-test) at p<.05.

Note: Data shown are for a single group of 14 irradiated and 16 sham-irradiated animals treated simultaneously and taken for analysis at intervals corresponding to 0.5, 1, 2 and 4 times the cycle period of seminiferous epithelium. The latter corresponds to one full cycle of spermatogenesis.

**8 HOURS OF CW-MWR
(SAR = 9.0 mW/g)**

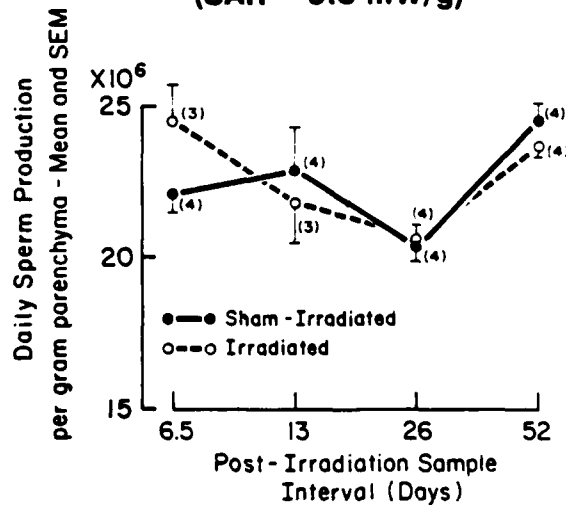


Figure 1. Calculated DSP/g obtained at fixed intervals following MWR exposure in a single 8-h session at 9 mW/g (CW). Here, and in the figures that follow, the numbers of animals in each of the specific sample subgroups are indicated in parentheses.

**8 HOURS OF CW-MWR
(SAR = 9.0 mW/g)**

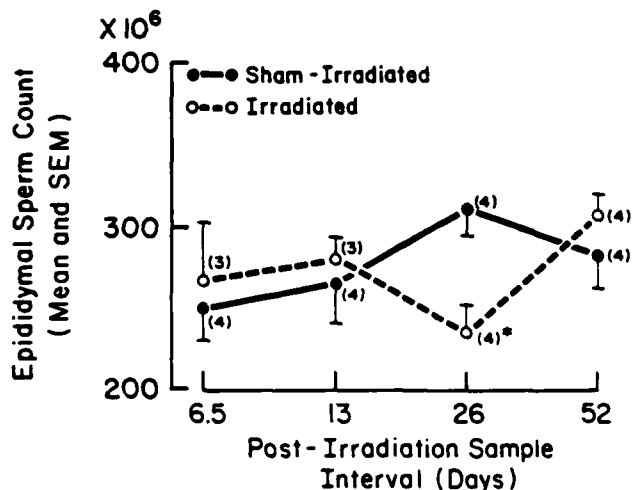


Figure 2. Epididymal sperm count for the same animals of Figure 1. The difference between group means at the 26-day posttreatment sample interval was significant in isolation; however, ANOVA applied to the entire experimental group did not reveal a significant difference due to treatment.

irradiation. We must conclude from the above data that no substantial neuroendocrine effect was produced by the MWR treatment.

The time course of rectal temperature during irradiation at the 9 mW/g dose rate is shown in Figure 4. Surrogate animals, of the same strain and body mass as used to obtain the above physiologic data, were used to obtain this thermal profile. Animals were irradiated using identical procedures and body temperature was sampled periodically using a rectal probe thermistor. The data indicated that equilibration at an elevated core temperature (40-41°C) was achieved by 1 h of irradiation and remained stable thereafter. Since the animals were removed from the waveguide periodically for insertion of the rectal probe, and since a slight decline in temperature of the sham-irradiated animals was evident, the MWR-induced whole-body thermal effects of Figure 4 must be construed as a conservative, underestimate of the actual temperature rise experienced by the animals in the primary experimental group. As shown in Figure 4, the net MWR-induced increment at this dose rate was on the order of 3°C. This dose rate was achieved after 1 h and remained stable for at least 8 h of exposure. This dose rate was, therefore, unambiguously thermogenic.

We did not attempt, at this point, to determine testicular temperature in the mobile rat, although it is now clear that this could be done. Neither was it our intention to examine whether or not orientation with respect to the MWR was actually part of the behavioral strategy of the rat; rather we concentrated the consequences of removing this option. In the waveguide chamber exposure system, quite stable regional SARs could be induced if the animal was made immobile by a general anesthetic. Despite the obvious complications of

body temperature regulation, drug interactions and so forth, it was felt that this procedure, if accompanied by rigorous thermal control studies, would be a useful approach to the question of the relationships between MWR exposure, testicular temperature rise, and the induced physiological decrement in spermatogenesis.

Acute Microwave Exposure of the Anesthetized Rat

The MWR dose rate required to achieve a prompt elevation of testicular core temperature above 39°C was lethal to most anesthetized animals when maintained for 60 min. However, it must be emphasized that the aims of these experiments were (1) to compare the MWR-induced thermal decrement in testicular function with that of conventional heating as well as (2) to explore the possibility of damage profiles that are more complex or perhaps inconsistent with a purely thermal underlying cause. To achieve the high testicular temperature necessary for an evaluation of point (1), we developed a two-stage exposure protocol. In subsequent experiments using considerably lower MWR dose rates, we approached point (2), namely, the correspondence between MWR and conventional thermal damage thresholds.

With the animals anesthetized and their hindquarters facing the energized end of the waveguide, a whole-body dose rate of 10.5 mW/g (1.3 GHz, PM-MWR) was maintained for 30 min. The dose rate was thereafter reduced to 7.7 mW/g for 60 min. A group of surrogate animals (n=9) was used for the determination of testicular and rectal temperature elevation during such exposure. These data are summarized in Figure 5. Note that the thermal equilibration time of the testis was between 15 and 30 min. Hence, the total irradiation interval was fixed at 90 min, with the first 30 considered as an

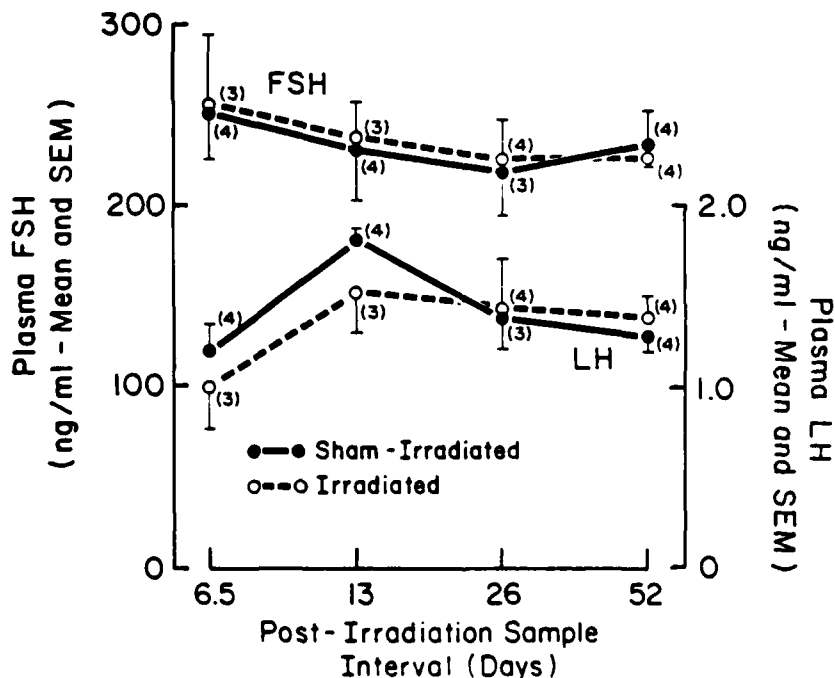


Figure 3. Circulating FSH and LH levels in plasma taken at the time of sacrifice for testicular examination (animals of Figs. 1 and 2). The data are within the expected range for normal animals of their respective ages.

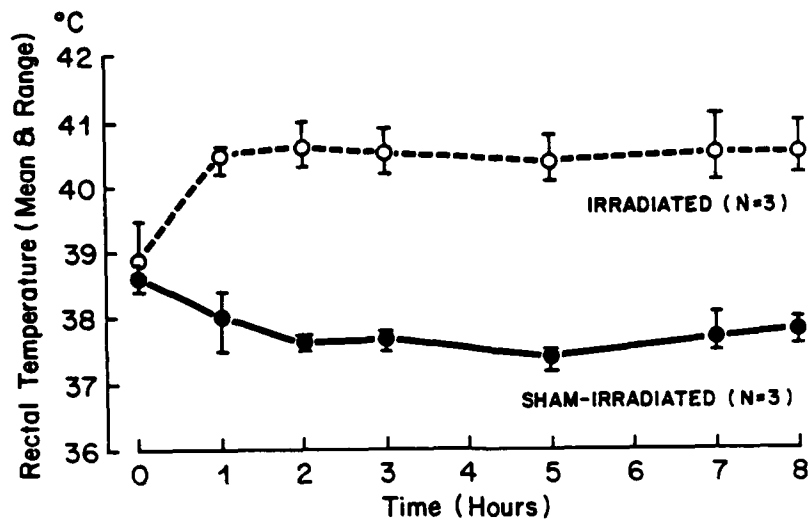


Figure 4. Rectal temperature in a group of 3 irradiated and 3 sham irradiated animals under the exposure conditions of the primary treatment group of Figures 1-3 (9 mW/g CW MWR). Temperature data was obtained by insertion of a lubricated deep rectal probe during a brief withdrawal of the animal from the waveguide.

**REGIONAL TEMPERATURES DURING IRRADIATION
TWO-STAGE PM-MWR - 10.5/7.7 mW/g**

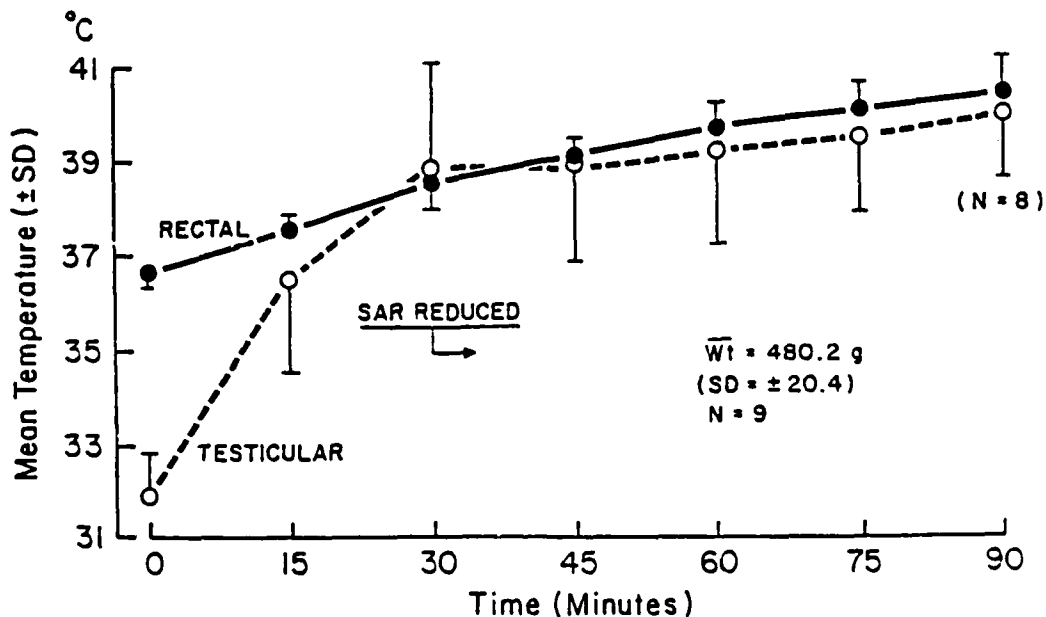


Figure 5. Deep rectal and intratesticular temperatures in anesthetized rats during MWR exposure at 10.5 mW/g (30 min) followed by 7.7 mW/g (60 min). Indwelling MWR transparent thermal probes (Vitek) were used to determine each; the animals remained anesthetized and within the waveguide throughout. These data were derived from nine animals (final reading omitted from one, as noted). Placement of the testicular transducer was verified in each instance by surgical excision at the conclusion of the experiment.

extended equilibration phase. In view of the heat exchange between testes and body through blood circulation, a calculation of regional (testicular) SAR was not attempted from these data.

The decrements in testicular function subsequent to such a two-stage MWR exposure protocol are shown in Figures 6 and 7. DSP/testis, epididymal sperm count, DSP/g parenchyma, and total testicular mass all showed a significant treatment effect (two-way ANOVAs yielded $p=.00001$, $.00002$, $.00068$, $.00003$, respectively). Statistically significant differences between the individual group means at each of the posttreatment intervals (SNK $p<.05$) are denoted on the graphs. Epididymal sperm count and DSP/testis (Fig. 6) each showed their maximal decrement at 26 days posttreatment, which correlated with the reduction in total testes mass (Fig. 7A). Sample group means of mass normalized potential sperm production (Fig. 7B) were significantly different only at the 13-day posttreatment sample. Within the irradiated subgroup, there was no statistical evidence for a difference among the means of the four postirradiation treatment intervals.

There was no indication (SNK) that rats in the treatment and control subgroups were significantly different at 52 days with respect to DSP, epididymal sperm or testes mass. However, the data indicated that at 6.5 days there were substantial decrements and that at 52 days posttreatment the means of the irradiated group did not exceed those at 6.5 days. A persistence of the effect of this relatively intense MWR exposure beyond one spermatogenic cycle must, therefore, not be ruled out. It was clear that this treatment had a significant and not necessarily reversible effect on testicular parameters. As shown below, lower MWR dose rates did not yield any detectable decrements at 6.5 days following exposure. Further study with a larger number of subjects for each sample point would be required to reduce overall variance and substantiate a persistent decrement.

Seminal vesicle mass (indicative of circulating testosterone levels) did not appear to show any main or treatment-dependent interaction effects under the two-stage MWR exposure protocol. There were other indications, however, of a significant change in neuroendocrine function (Fig. 8). Two-way ANOVA indicated a significant difference in FSH levels due to irradiation ($F(1,37)=7.86$; $p=.0079$). This difference appeared to derive largely from elevations of FSH at 26 and 52 days posttreatment. An overall treatment-dependent difference in LH levels was also found ($F(1,35)=7.45$; $p=.0096$).

Additional experiments were carried out at dose rates of 7.7 and 4.2 mW/g (1.3 GHz, PM-MWR) so as to gain some estimate of the threshold for induction of a significant MWR-induced deficit. At these lower MWR dose rates exposure durations of 60-90 min were consistent with long-term survival of the irradiated animals. The rectal and intratesticular temperature curves obtained from weight-matched surrogate animals during 90-min exposure at these dose rates are shown in Figure 9.

Again, the testicular thermal time constant was determined to be on the order of 30 min. With 7.7 mW/g, intratesticular temperatures rose to levels in excess of 39°C . Mean testicular temperature did not exceed 36°C during exposure at 4.2 mW/g. We would expect, therefore, that if thermal factors only are considered, the latter dose rate should be below threshold. The effects on testicular function of MWR treatment with similar (within 2%) SARs are compared in Figures 10 and 11.

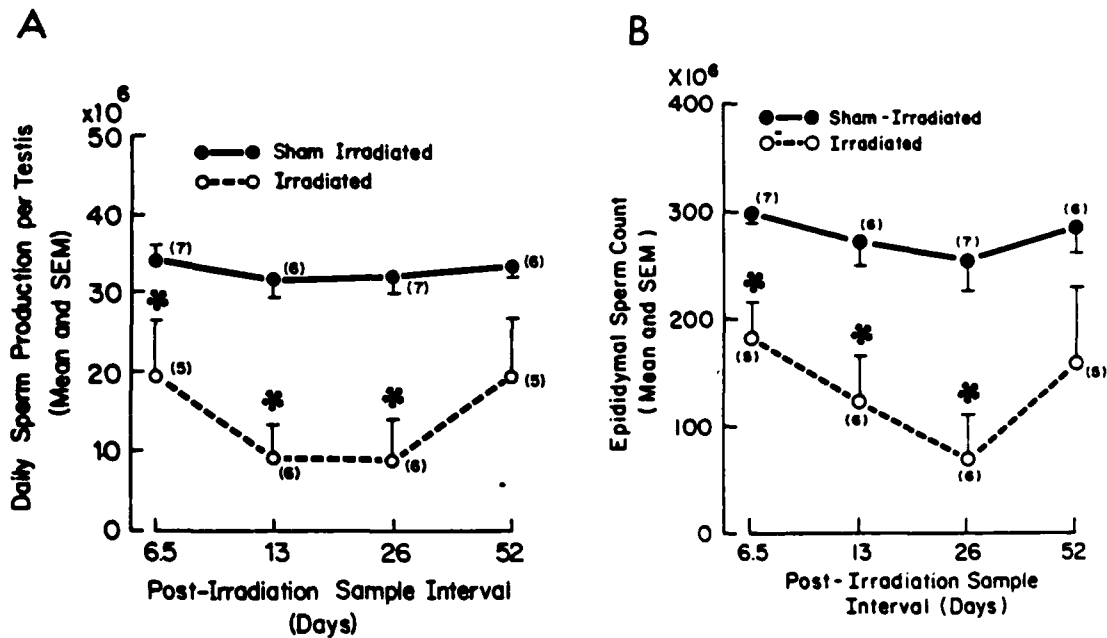


Figure 6. (A) Calculated DSP per testis and (B) epididymal sperm count subsequent to irradiation protocol of Figure 5, as a function of posttreatment interval. Here (and in the figures that follow) instances of significant differences between sham (control) and actual irradiated group means are indicated by "*" (a posteriori demonstrations of a significant treatment dependent effect from ANOVA).

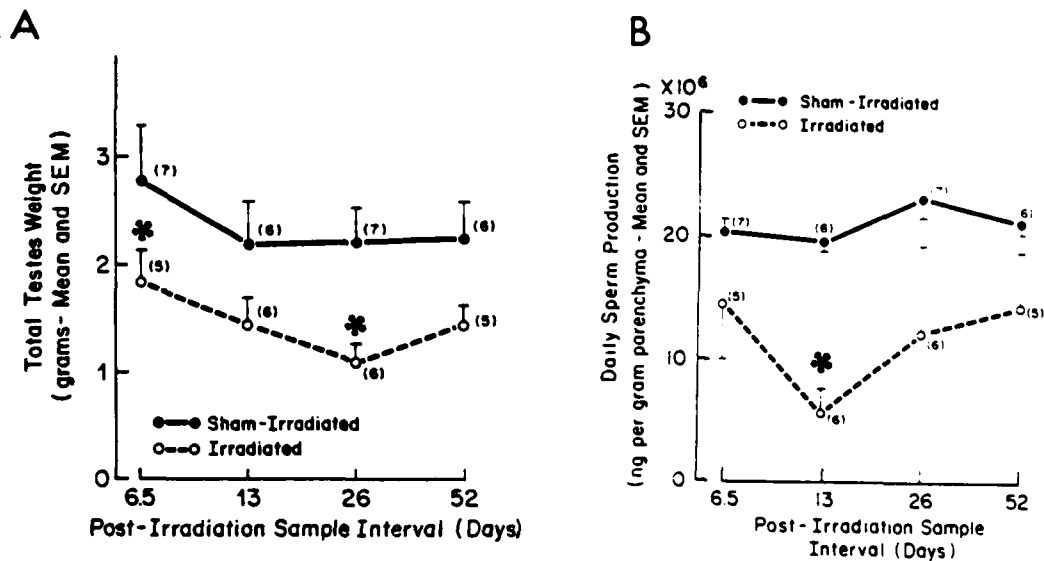


Figure 7. (A) Sum of left and right testis mass and (B) mass normalized potential DSP for animals of Figure 6.

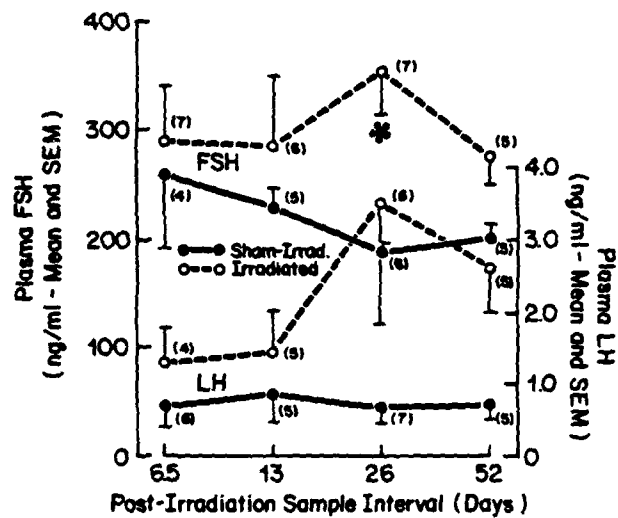


Figure 8. Plasma FSH and LH as a function of postirradiation sample interval. The data are from the same animals as Figures 6 and 7. The data from 5 animals (3 sham, 2 irradiated) fell outside of the accepted RIA range and were omitted from this graph.

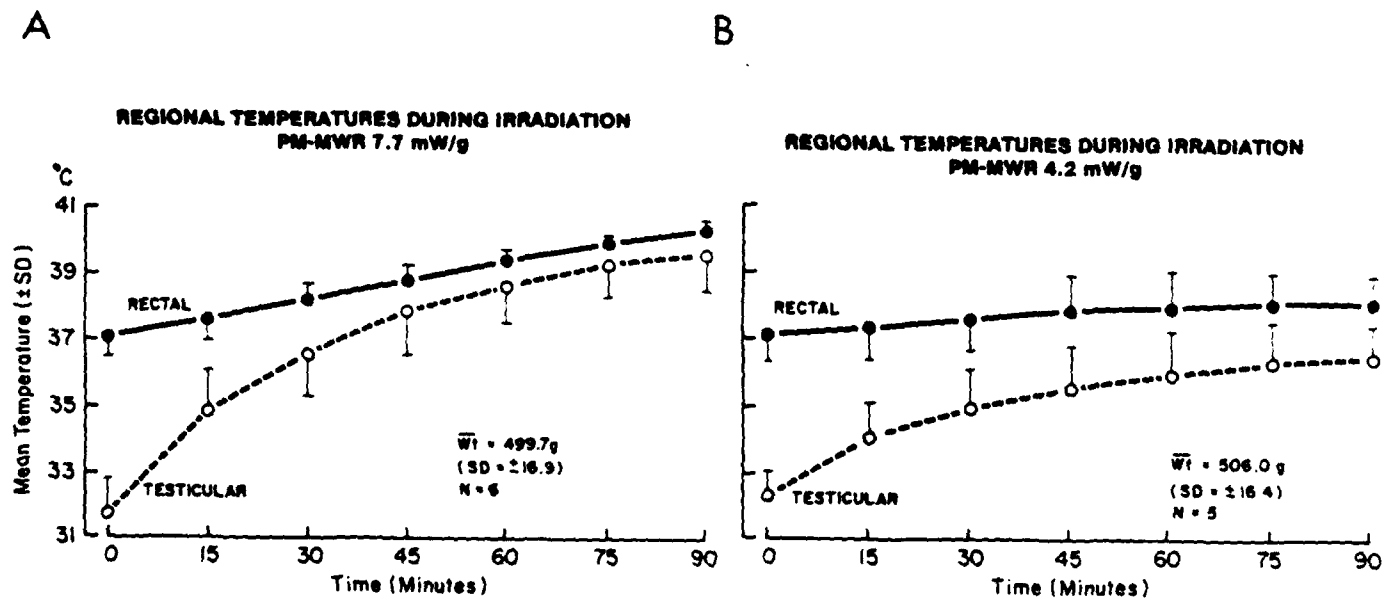


Figure 9. Deep rectal and intratesticular temperature at SARs of (A) 7.7 mW/g (n=6) and (B) 4.2 mW/g (n=5).

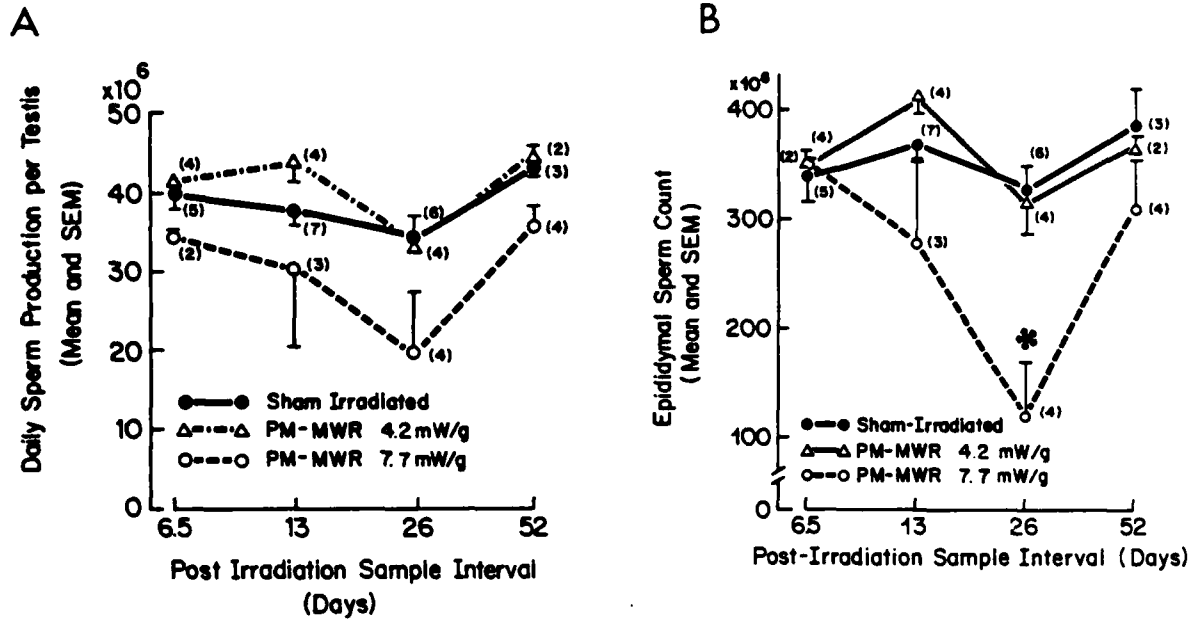


Figure 10. (A) Calculated potential DSP per testis and (B) epididymal sperm count subsequent to MWR exposure at 4.2 and 7.7 mW/g. See Figure 6 legend for additional details.

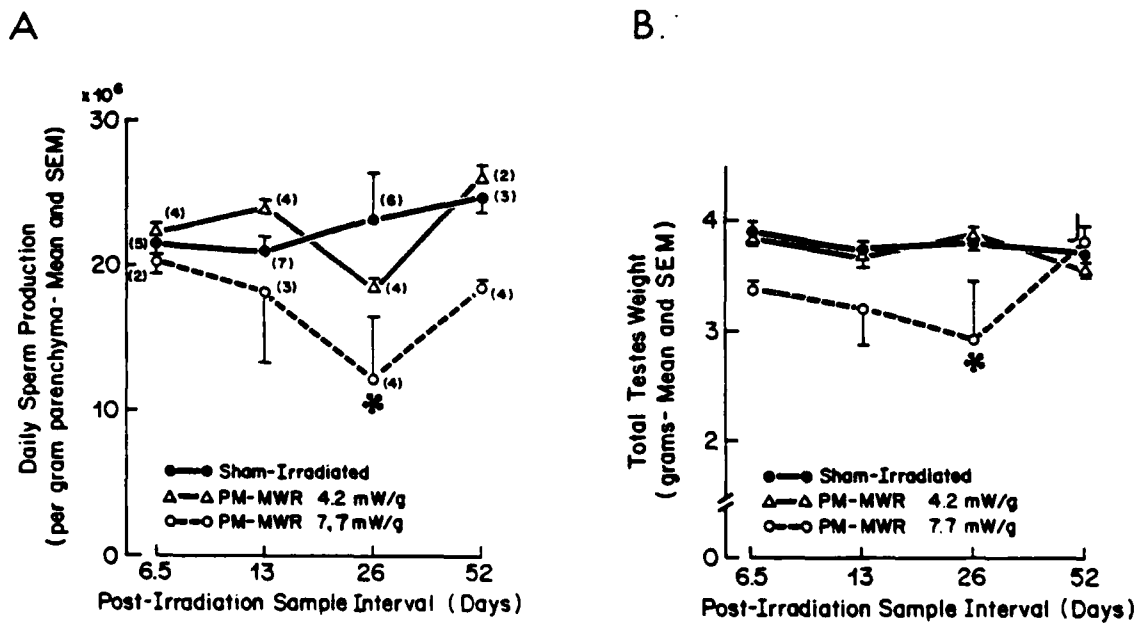


Figure 11. (A) Summed right and left testis mass and (B) mass normalized potential DSP for the animals of Figure 10.

At an SAR of 7.7 mW/g there were significant MWR-induced reductions in calculated potential DSP/testis, DSP/g parenchyma, epididymal sperm count, and total testes mass (two-way ANOVAs yielded $p=.00142$, $.00508$, $.0008$ and $.00342$, respectively). The overall treatment difference appeared to originate from decrements that appeared most clearly in the late posttreatment sample, specifically, at 26 days (SNK, see figures). Testes mass was significantly reduced at 6.5 days as well as at 26 days. At the other postirradiation sample intervals, mean total testicular mass, while quantitatively smaller, was not statistically different from control values. The prompt reduction in testicular mass subsequent to 7.7 mW/g was similar to that observed with the two-stage exposure protocol (cf. Fig. 7A).

Within the 7.7 mW/g irradiated subgroup, calculated DSP/testis and total testes mass mean values at 26 and 52 days posttreatment were statistically different (SNK, $p<.05$). Also, epididymal sperm count in this subgroup was significantly lower at 26 days than at any of the other three posttreatment intervals.

At 4.2 mW/g, on the other hand, no significant difference between control and irradiated group could be found for any of these measured testicular parameters (cf. Figs. 10 and 11; two-way ANOVAs each yielded $p>.1$).

Plasma FSH and LH data presented rather large variance at the 7.7 and 4.2 mW/g SAR; no statistically significant effects of either could be detected. These data are not shown, since taken out of context, the large variance could be misinterpreted.

In summary, there was a weak effect of the 90-min exposure at 7.7 mW/g, as demonstrated by significant decrements in testicular function most marked at a latency of 26 days. We must conclude that 7.7 mW/g was at or very near the threshold dose rate and that 4.2 mW/g was subthreshold for producing a significant effect on key parameters of testicular function.

Comparison of Acute CW and PM MWR Exposure

The above data were obtained using PM MWR with a low duty cycle (.0006), hence, a large ratio of peak to average power density. It was of interest, therefore, to determine next whether similar results would follow from exposure to CW MWR.

The testicular and rectal temperature profiles obtained during irradiation of anesthetized rats for 90 min using 1.3 GHz CW MWR at 7.5 mW/g are shown in Figure 12. Note that this profile was virtually identical to that obtained with PM MWR of comparable intensity (Fig. 9A); the mean temperature shift induced by PM MWR was virtually identical to that obtained with CW MWR at every sample point beyond 15 min of exposure. This profile is the expected result since the whole-body thermal time constant, or even that of the testis in isolation, is orders of magnitude longer than the MWR pulse width (26).

PM and CW MWR at this SAR each yielded significant overall group differences in the calculated DSP, number of epididymal sperm and testes mass. A summary of the resulting statistical data is given in Table 2. Note that

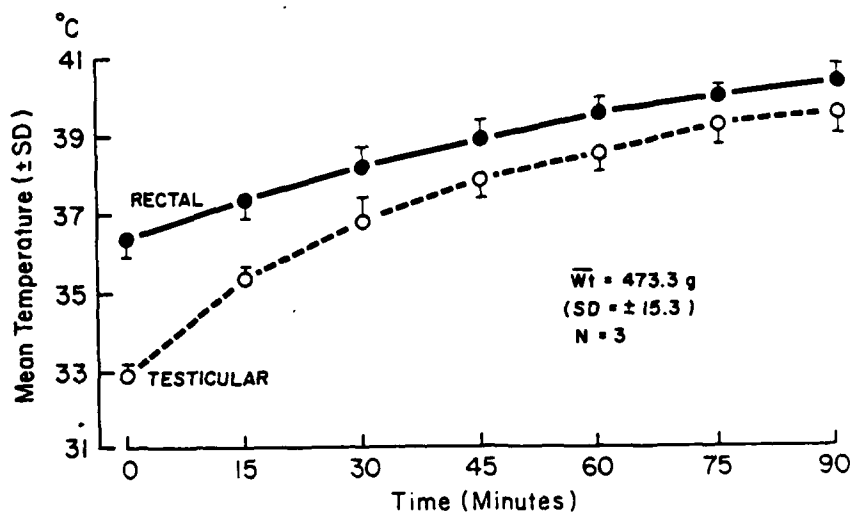


Figure 12. Rectal temperature and temperature at the center of the testis in anesthetized rats as a function of time during exposure to MWR at 7.5 mW/g.

TABLE 2. STATISTICAL COMPARISON OF THE RESULTS OF EXPOSURE TO PM AND CW MWR AT 7.5 MW/G - THE RESULTS OF TWO-WAY (TREATMENT AND POSTTREATMENT SAMPLE INTERVAL) ANOVA.

VARIABLE	PM MWR vs. Controls	CW MWR vs. Controls	PM MWR vs. CW MWR
DSP/g	F(1,28)= 6.903 p=.0132	F(1,28)= 2.359 p=.1323 (ns)	F(1,18)= 1.177 p=.2925 (ns)
DSP/testis	=10.301 =.0036	= 4.345 =.0439	= 1.520 =.2318 (ns)
Epidid. Sperm	=15.078 =.0009	= 7.299 =.0112	= 1.865 =.1963 (ns)
Testes mass	=13.153 =.0015	= 4.723 =.0363	= 2.732 =.1124 (ns)
Seminal ves.	= 3.063 =.0876 (ns)	= 0.003 =.9526 (ns)	N.A.

seminal vesicle mass was not significantly affected and that mass normalized DSP showed a treatment effect for PM but not CW MWR.

Closer examination of the sample group means suggested that the observed treatment effect derived largely from decrements at 26 days following treatment. Figure 13 shows the number of epididymal sperm and Figure 14 the calculated mass-normalized DSP as functions of the interval between irradiation and their determination. With both PM and CW MWR the reduction of epididymal sperm at the 26-day posttreatment sample point was marked by a substantial return to near control levels by 52 days. Mass normalized DSP, while apparently reduced in both treatment groups at this same latency, yielded a statistically significant decline only in the PM treatment group (see Table 2). The DSP/testis data (not shown) were essentially the same.

Due to the large variance in the testis mass of the treatment groups (Fig. 15), the substantial decline observed at 26 days posttreatment was not statistically significant in isolation. ANOVA did confirm, however, that testis mass in both the PM and CW treatment groups differed from controls (cf., Table 2). A decline with partial but no firm statistical indication of a recovery after one full spermatogenic cycle seems to be the net effect on this variable. The data would suggest that PM but not CW MWR yielded an immediate (within 6.5 days) decline in total testes mass at this dose rate. However, the apparent overall differences in testes mass of the corresponding PM and CW irradiated subgroups were not statistically significant ($F(1,18)=2.732, p=.1124$).

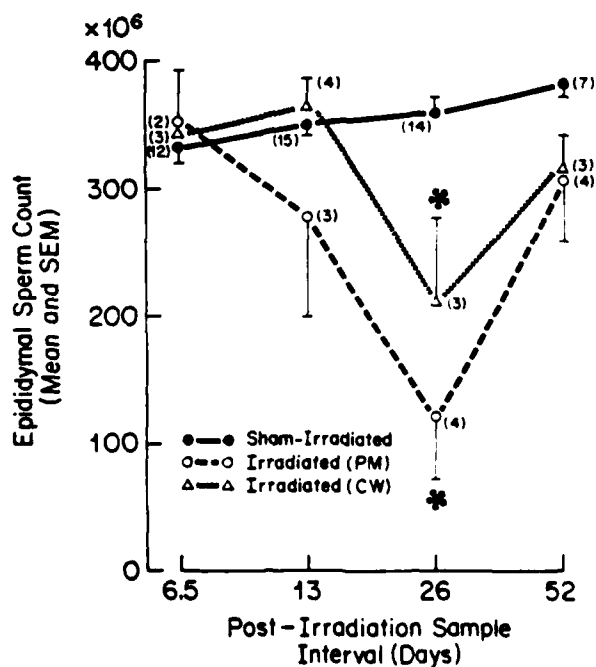


Figure 13. Comparison of the changes in epididymal sperm count following PM or CW MWR at 7.5 mW/g.

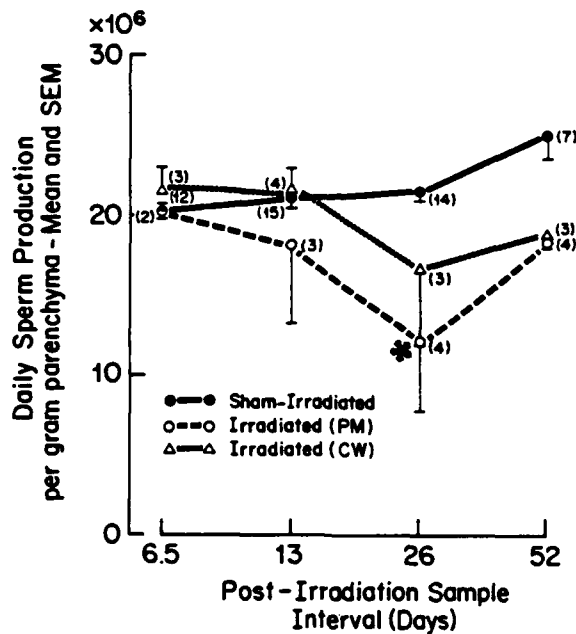


Figure 14. Comparison of the changes in calculated DSP following PM or CW MWR at 7.5 mW/g.

Acute Conventional Heating

As shown in Figure 16, testicular temperature (measured at the center of the testis) reached equilibrium with the water bath within 15 min of immersion. A thermal time constant of approximately 5 min appeared to best characterize the equilibration process (cf. Fig. 16A,D, wherein data were obtained at a finer time scale). Since this equilibrium temperature was essentially that of the water bath, we can assume that there were no significant temperature gradients across the testicular cross section. Even with a sustained difference between subjects' rectal temperatures of more than 1.5°C testicular temperature equilibrated to within $.25^{\circ}\text{C}$ of the waterbath (cf., Fig. 16B).

Figure 17 shows the calculated potential DSP and epididymal sperm count at the intervals of 6.5, 13, 26 and 56 days following immersion for 60 min at the indicated temperatures. Of the group of animals subjected to testicular immersion at 43°C for 60 min, more than half died before physiological data could be obtained. This subgroup, therefore, was not included in our analysis.

The temperature effect was highly significant (DSP/g - $F(2,34)=176.1$, $p<.00001$; epididymal sperm - $F(2,34)=179.8$, $p<.00001$). A marked general decline without recovery was seen after treatment at 41°C . Immersion at 39°C , on the other hand, had marginal and unclear effects on calculated DSP/testis and on epididymal sperm count. A decrement at 6.5 days (S1) was tentatively identified. However, statistical comparison of the individual subgroup means (SNK) did not reveal any significant differences between immersion at 39 and 34°C (control).

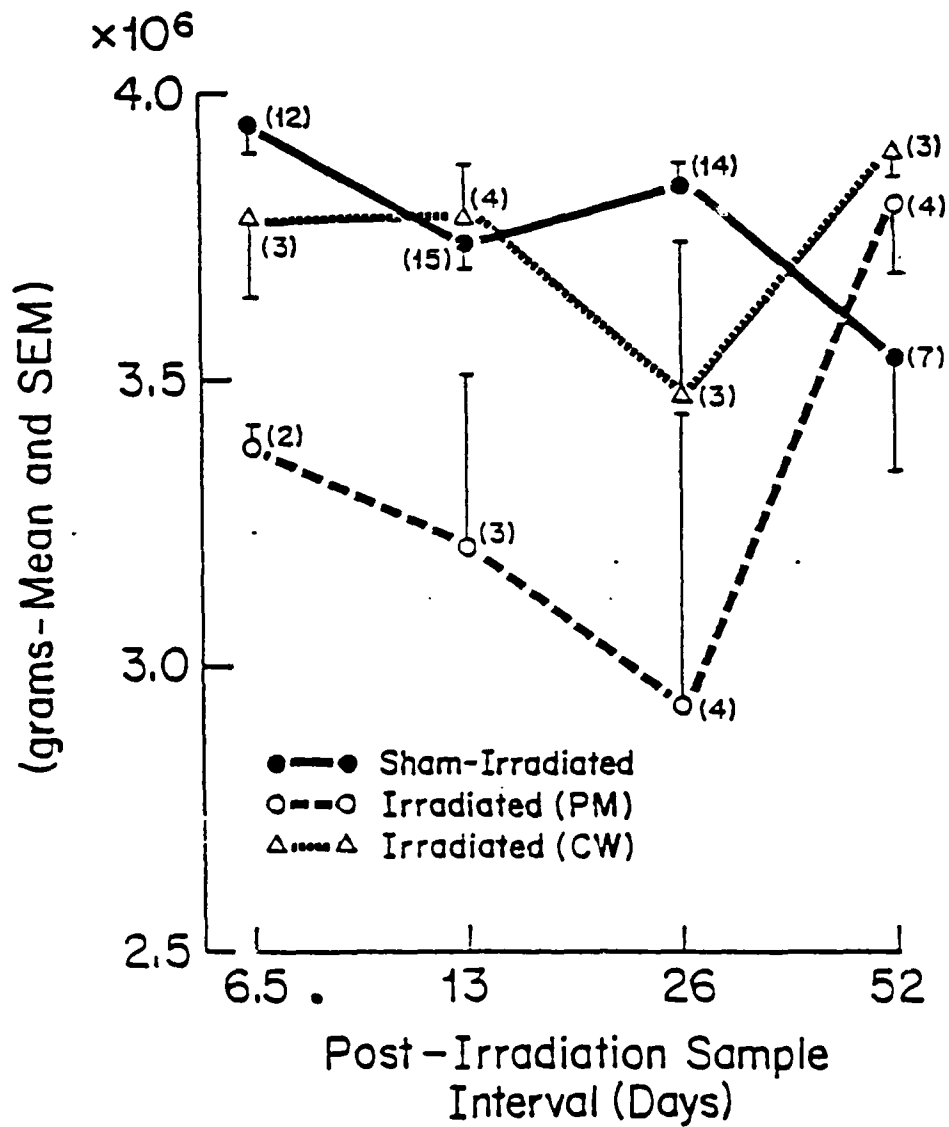


Figure 15. Comparison of the changes in summed mass of right and left testis following PM or CW MWR at 7.5 mW/g.

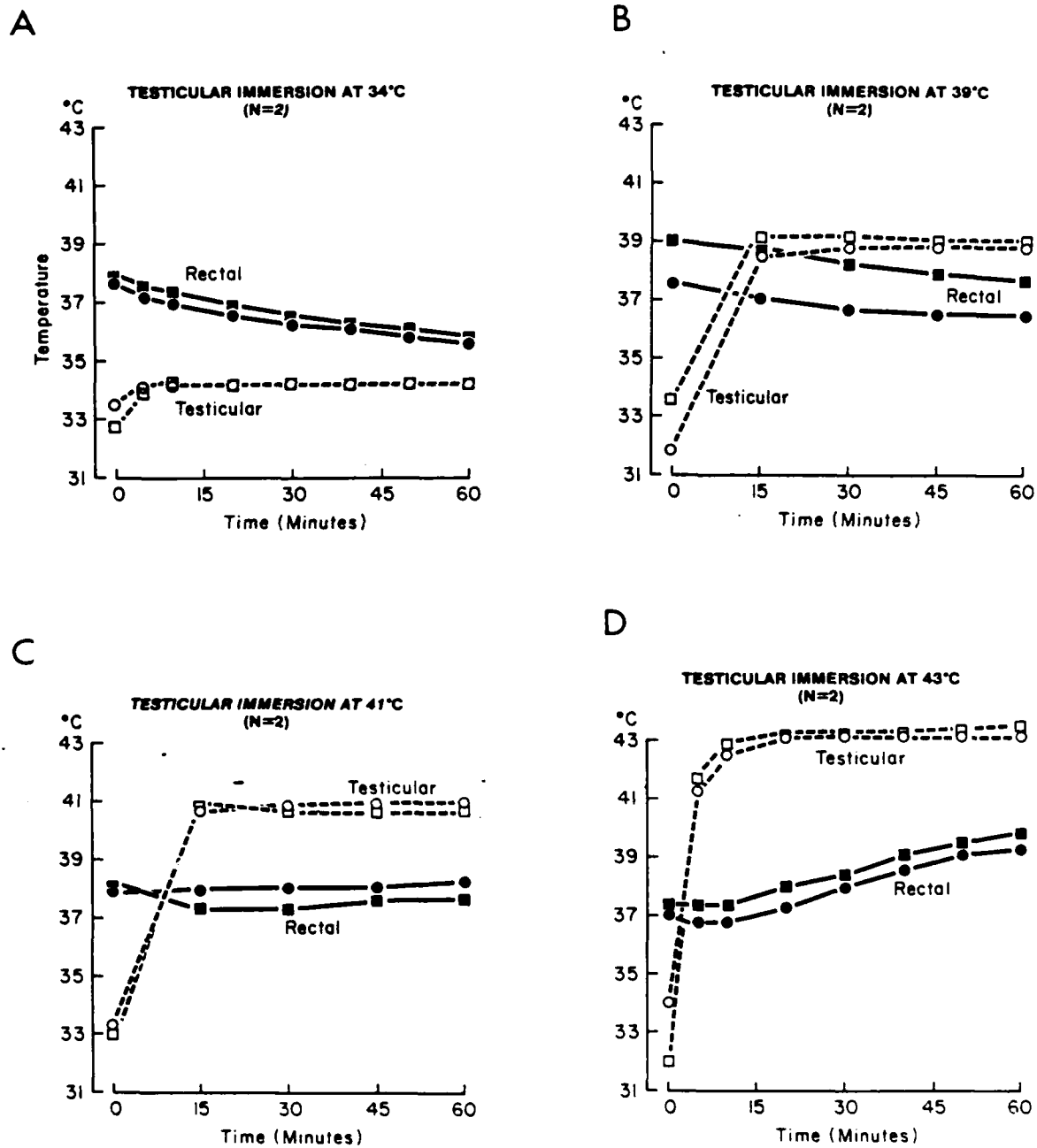
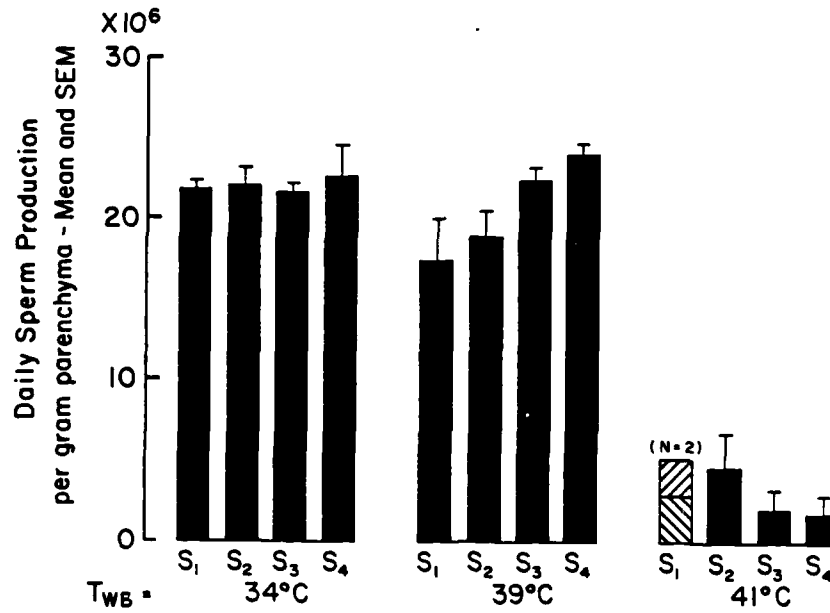


Figure 16. Core temperature and temperature (continuously monitored) at the center of the testis as a function of time, during immersion of the testicles in water at four different, constant temperatures. Core temperature was taken with a deep rectal thermistor. Testicular temperature was obtained by a fine, silicon-encapsulated bead thermistor thrust through a needle channel to the testicular core. Placement of the latter was verified in each instance by surgical excision at the conclusion of the experiment. Two animals were adequate for each of the temperature runs in view of the very slight experimental error with this technique.

A



B

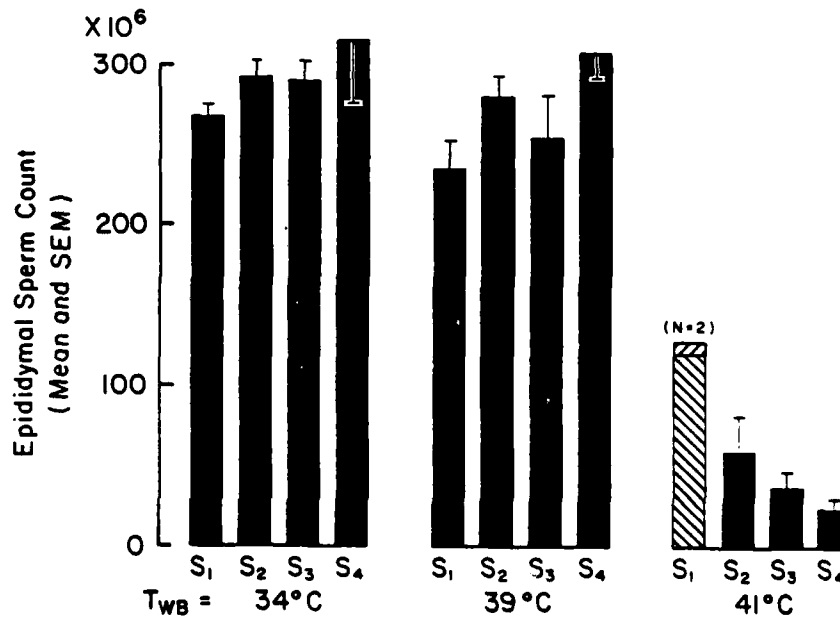


Figure 17. (A) Potential DSP and (B) epididymal sperm count as a function of immersion temperature at four different intervals subsequent to treatment: S₁, S₂, S₃, S₄ correspond to 6.5, 13, 26 and 52 days posttreatment, respectively. Statistical significance discussed in text. Four animals per sample point, except where noted (46 animals total).

The effects of constant temperature immersion of the testicles on plasma FSH and LH are shown in Figure 18. An elevation of both of these measures was quite evident after immersion at 41°C (FSH: $F(2,34)=13.56$, $p=.00014$; LH: $F(2,32)=14.52$, $p=.00011$; several outliers were cast out in each instance). However, a specific effect of immersion at 39°C was doubtful. While further statistical tests were justified and a two-way ANOVA comparing the 34 and 39°C data specifically verified a significant overall elevation in LH ($F(1,22)=5.60$, $p=.0258$), no group mean difference at any specific posttreatment interval could be established by the SNK test. The threshold for a thermally induced elevation of FSH (60-min immersion) was unambiguously above 39°C.

From these data it appeared that with the temperature held at an elevated, stable level for the period of time practical under a single dose of barbiturate anesthesia, the testicular temperature would have to achieve, and more probably exceed 39°C in order to obtain detectable changes due to temperature alone.

MWR-induced Enhancement of Sperm Abnormalities

Using relatively gross morphological criteria (23), the fraction of abnormal epididymal sperm was determined from a sample of 200 sperm in each animal. Subsequent to exposure using the two-stage protocol (11.4 followed by 8.2 mW/g PM MWR) some evidence for increased frequency of abnormality at 26 days posttreatment was apparent (Fig. 19). The irradiated subgroup yielded data with much greater dispersion than controls and two-way ANOVA did not confirm a statistically significant difference between the groups due to treatment ($F(1,38)=2.342$, $p=.1305$). These data must be viewed as highly suggestive, however, in view of the results from exposure to PM and CW MWR at lower dose rates.

The fractional sperm abnormalities detected following exposure to PM and CW MWR at 7.5 mW/g are shown in Figure 20. No overall difference between the treatment and control groups could be established (PM: $F(1,28)=2.491$, $p=.1222$; CW: $F(1,28)=2.325$, $p=.1350$). The CW group did show a significant interaction between treatment and posttreatment sample interval ($F(3,28)=3.112$, $p=.0414$); a similar interaction effect was only marginally significant in the PM subgroup ($F(3,28)=2.565$, $p=.0736$). For neither treatment were found significant differences between specific postirradiation sample group means found (SNK, $p>.05$).

Limited study of sperm abnormalities following exposure to lower levels of irradiation was also carried out. With CW MWR at 2 mW/g and with PM MWR at 4.7 mW/g the relative numbers of abnormal sperm were no different in irradiated than nonirradiated control animals. These data must be considered preliminary. Much more extensive experiments must be carried out before the null results at these lower levels, and the tentative positive result at 7.5 mW/g and higher, can be established with any certainty.

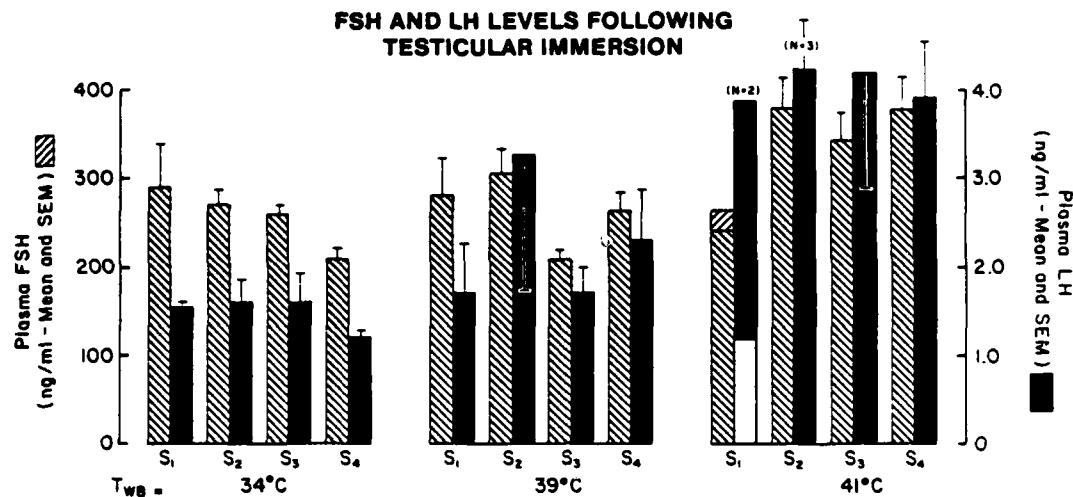


Figure 18. Plasma FSH and LH determined from trunk blood taken on termination day S1 through S4; same animals and sample points as in Figure 17. Four animals per sample point, except where noted (45 animals total).

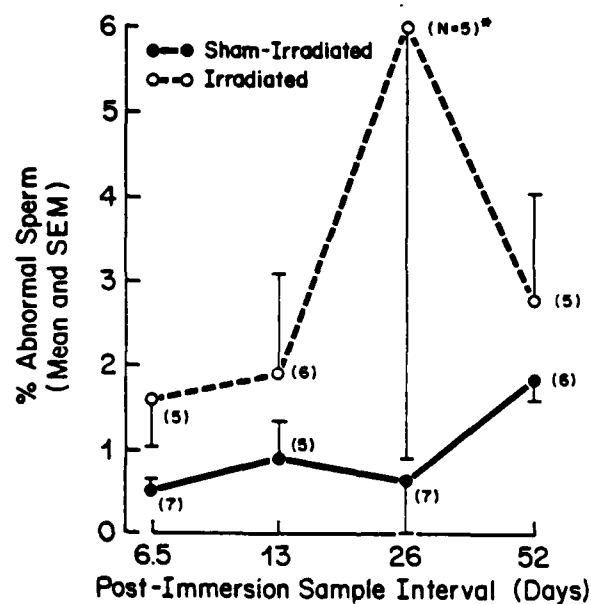


Figure 19. Fractional number of abnormal epididymal sperm found after two-stage PM MWR exposure (30 min at 11.4 mW/g followed by 60 min at 8.2 mW/g) of anesthetized rats, as a function of posttreatment sample interval.

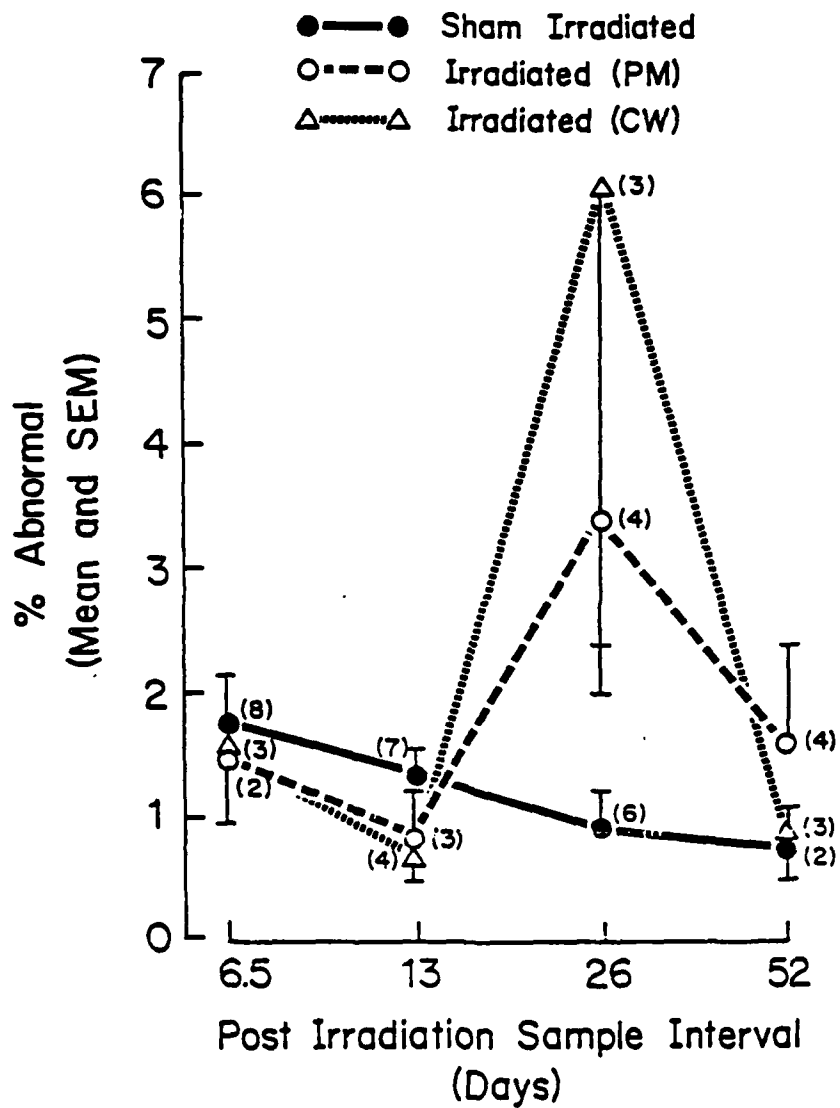


Figure 20. Comparison of the fractional number of abnormal epididymal sperm found after 90 min PM versus CW irradiation of anesthetized rats, as a function of postirradiation sample period. Statistical evaluation in text.

DISCUSSION

We have made use of quantitative expressions of spermatogenesis for a more direct evaluation of the functioning of the germinal epithelium. The determination of potential DSP at discrete posttreatment latencies appears to be an efficient and sensitive method with which to quantify the differential sensitivity of germ cells to MWR exposure at particular stages of maturity.

At an SAR of 9 mW/g, sustained for 8 h, the suggestive decline in epididymal sperm count 26 days after treatment reflected the threshold decrement induced by the MWR on primary spermatocytes for the most part. A differential sensitivity of germ cells at this stage of maturation has been reported with respect to conventional heating of the testes (17). The other measures of testicular function did not confirm, however, the existence of a significant effect on the mobile rat at this MWR dose rate. In view of the large number of variables measured, we must view the reduction in epididymal sperm count at 26 days posttreatment as highly questionable. Further, in view of the unarguable heat-sensitivity of the testis (17,20) we must conclude that even our clearly thermogenic dose rate was not sufficient to induce a critical temperature rise in the testes of unrestrained rats. In this respect our results are in essential agreement with those of Berman et al. (3). These data offer no support for macroscopically athermal effects on the testes.

However, the regional variations in absorbed MWR energy accompanying whole-body exposure are well known (cf. Ref 27). Further, due to the volume heat production characteristic of MWR absorption, conventional heating does not always yield a comparable internal temperature distribution. Since the focus of these studies was on testicular physiology, we emphasized a treatment protocol that yielded demonstrably steady state shifts in testicular temperature. Whole-body temperature, while monitored and an important measure of the likelihood of survival, was of secondary importance here. This is not to imply that only thermal factors were relevant, but rather to provide a relatively uniform set of conditions under which to evaluate the significance of an important physiologic variable.

In acute experiments using a two-stage irradiation protocol, we were able to produce a marked elevation in testicular temperature without an accompanying fatal rise in core temperature. The subsequent decrement in spermatogenesis and related variables was most evident at 13 and 26 days postirradiation, with some suggestion of a maximum effect detected at 26 days postirradiation. A significant decrement at 6.5 days following treatment was indicative of a direct impact of the irradiation on mature spermatids under this protocol. MWR at 7.7 mW/g appeared to very near threshold in these studies. Further, this level of exposure was correlated with a rise in the intratesticular temperature to levels close to the observed thermal damage threshold (cf. Figs. 9A and 17). The appearance of the most significant decrements at 26 days posttreatment is in agreement with such conventional heating experiments as have been carried out under comparable protocols (17,20,28). Namely, conventional heating to this extent for 60 to 90 min has its most prominent effect on maturing germ cells in the stages from early

spermatids to premeiotic spermatocytes; this would be expressed as a reduction in potential DSP observed 15-35 days posttreatment.

Our MWR results differ from the conventional heating findings in that we do not find the expected recovery to control values after one full spermatogenic cycle (52 days; cf Figs. 6-7, 10-11, and 13-14). While the number of animals studied was large, the size of the 52-day posttreatment subgroup was less than satisfactory for definitive statistical treatment.

On the other hand, PM MWR at 4.2 mW/g clearly must, in a statistical sense, be viewed as subthreshold as a consequence of these studies. Such an exposure yielded a 4° to 5°C rise in testicular temperature after 90 min. It is worth noting that little or no decrement in testicular function could be seen following conventional heating to levels even several degrees higher for a comparable length of time (Fig. 17; also see Refs. 20,28). These data provide further support for the hypothesis of an essentially thermal mode of interaction of MWR with testicular function.

In the anesthetized rat, the threshold for a decrement in testicular function due to temperature elevation was found to be between 39 and 41°C for 60 min using conventional heating. It was essential that our methodology be adequate to detect the decrement in testicular function that would follow from such a brief exposure to moderate temperature elevation. At temperatures substantially in excess of 41°C, we could not exclude the possibility of significant damage to other organ systems, as suggested by the lethality of testicular immersion at 43°C for 60 min. These positive control data thus show that, although athermal MWR effects are not excluded, our procedures had the requisite sensitivity for useful characterization of a null as well as positive result of MWR exposure.

Irradiation with PM and CW MWR at 7-8 mW/g appeared to yield quite similar results. Each of the major, and for the most part only, significant differences between the PM and CW treated groups and controls was evident at 26 days following treatment. Again, we can work backwards and identify germ cells in the early spermatid or spermatocyte stage of development as the cell type chiefly affected. The similar effects of PM and CW MWR suggest that average and not peak power density was the critical factor. Further, the testicular temperature achieved under these exposure conditions and the correspondence of these data with that seen using comparable, conventional heating of the testis in an anesthetized preparation strongly suggest a thermal basis for these results. As suggested by experiments using isolated spermatid fractions (29), protein synthesis in this cell type is reduced at 37°C with respect to the levels found at normal scrotal temperatures (32-34°C). Under our exposure conditions testicular temperature exceeded this level for the final 60 of the 90 min exposure period.

Quantitative evaluation of the morphology of sperm morphology can be a useful indicator (23) of otherwise nonspecific reproductive stress. The sample sizes attained in these studies were not sufficient to permit an unambiguous determination of MWR threshold for such changes. It seems fair to say that the threshold for production of an elevated frequency of abnormality in epididymal sperm was approximately that required for production of detectable decrements in DSP. Of course, abnormalities would potentially have a much greater impact on subsequent generations than a simple decline in sperm count. A caution is in

order, therefore, with respect to using the sperm reduction potential of MWR exposure of the testicles as a method for "temporary" sterilization (30).

While not a conclusive proof, the experiments performed to date strongly suggest a classically thermal basis for both the threshold level and the differential sensitivity to MWR of germ cells at specific stages of maturity. In view of the relatively higher sensitivity of early spermatids and spermatocytes to conventional heat, we have a ready means to interpret the similar findings following MWR exposure. Rather than arguing in favor of some "athermal" interaction that could depend upon the maturation state of the germ cells (such as meiotic versus premeiotic) such data tend to support the contention of a graded threshold thermal sensitivity of the seminiferous epithelium.

Since whole-body exposure protocols are frequently used as the basis for setting exposure standards, we must be certain that a systematic error in estimation of the proper upper bound for exposure is not introduced inadvertently. For example, with the unrestrained animal, despite the fact that the irradiation frequency of 1.3 GHz was near resonance for the rat and that the elevation in body temperature confirmed that substantial absorption of the energy was taking place, it was apparent that intact behavioral thermoregulatory strategies confounded any requirement for a fixed, known testicular dose rate. One can argue, therefore, that whole-body irradiation protocols designed for the unrestrained animal, while providing some useful information for estimations of potential exposure hazard, have done little to further our understanding of the locale and mechanism of potential interactions of MWR with spermatogenesis. More precise specification of the testicular dose rate is a minimum first step for this purpose. In addition to the physiological thermoregulatory mechanisms that may serve to blunt the expected rise in testis temperature at the expense of core temperature, the alert and active animal is free to adopt a thermal avoidance strategy such that the hindquarters are shielded by the remainder of the body mass during a significant fraction of the exposure period. Admittedly, this frames the issue along thermal lines, that is, whether MWR-induced deficits are indeed fully explicable on the basis of the testicular thermogenic consequences of such exposure. Nonthermal modes of interaction may be significant (e.g., ref. 30). One must acknowledge the quantifiable thermosensitivity of germinal tissue and work towards a definition of the three-way relationship between MWR exposure, testicular temperature, and testicular physiology.

The issue cannot be considered closed, however, until the possibility of fine scale frequency sensitivity is examined. An experimental verification of frequency specific "resonance" absorption of MWR by DNA has been reported (31). We would predict, on the basis of our data from acute exposure experiments, that if such resonance effects apply to irradiation in vivo and if thermalization (either macro- or microscopic) is the significant factor, then the major changes will be observed at a considerable latency (26 days) following exposure. There already is evidence (16, 32) that MWR-related chromosomal abnormalities in the mouse are induced primarily at the spermatocyte or late spermatid stage of maturation.

CONCLUSIONS AND RECOMMENDATIONS

Our conclusions can be stated as follows. For the various aspects of testicular function studied to date, the sensitivity of this organ system to MWR is approximately what one would expect from its known sensitivity to conventional forms of heating. While there are differences between the results of conventional and MWR heating that may relate to the intratesticular temperature profiles that are produced thereby, the functional implications of such differences are exaggerated by proximity of MWR dose or conventional temperature elevation to the threshold level necessary for evident decrement in function. When freely mobile, the adult male rat can sustain near lethal acute MWR dose rates without showing evident signs of testicular decrement in the short-term (6.5 days) or long-term (52 days) posttreatment. In the anesthetized preparation, wherein the testicular dose rate is held constant, the effects of acute MWR exposure appear to fairly reflect the sensitivity of that organ system to conventional heating. Secondary factors other than thermal gradients may play a role, such as heat exchange between testes and the rest of the body mass or rate of temperature rise, but there as yet appears no evidence to support a significant factor other than classical, macroscopic temperature rise within the testis at this single irradiation frequency. At SARs in the range of 7-8 mW/g an incidence factor must be taken into account, in that the prolonged expression of testicular damage (at from 6.5 to 52 days posttreatment) may demonstrate a sharp threshold over the exposed population with some animals showing a total lack of decrement possibly due to a marginally smaller testicular SAR.

Now that we can confidently establish a posttreatment sample interval of on the order of 26 days as providing the most sensitive end points, detailed parametric study should proceed and with greater efficiency. For testicular function, the critical factors may not include whole-body dose rate as such. We recommend that irradiation frequency (because of possible resonance effects (1) within the testis itself or (2) with DNA molecular structure), direction of incidence and secondary influences (as vis-a-vis drugs or physiologic state) on testicular heat exchange should be the primary factors to consider in future efforts to evaluate MWR-induced changes in testicular function.

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