ABSTRACT

The response of mouse testis stem cells to hyperthermia and combined hyperthermia-radiation treatments was assayed by spermatogenic colony regrowth, sperm head counts, testis weight loss, and fertility. With the use of spermatogenic colony assay, thermal enhancement ratios at an isosurvival level of 0.1 were 1.27 at 41°, 1.80 at 42°, and 3.97 at 43° for testes exposed to heat for 30 min prior to irradiation. Sperm head counts were reduced by heat alone from a surviving fraction of 0.58 at 41° to 0.003 at 42.5-43.5°. Curves for sperm head survival measured 56 days after the testes had been heated for 30 min prior to irradiation were biphasic and showed a progressive downward displacement to lower survival with increasing temperature. The 41, 42, and 43° curves were displaced downward by factors of 2, 58, and 175, respectively. The proportion of animals remaining sterile after 30 min of heat (41-43°) and the median sterility period in days increased with increasing temperature. The minimum sperm count necessary to regain fertility was 13% of the normal mouse level.

INTRODUCTION

Recent clinical and laboratory studies have restimulated interest in treating cancer with hyperthermia alone or in combination with radiotherapy or chemotherapy. Pettigrew et al. (14) reported the use of whole-body hyperthermia in patients in the terminal stages of cancer with relatively few major complications while Cavaliere et al. (1) and Stehlin (19) encountered several major normal tissue complications after heating cancers of the limb with isolated perfusion. Obviously, when tumor tissues are heated, normal tissues will also be exposed. One of the primary concerns of whole-body hyperthermia, in particular, has been that of critical organ tolerance, but even with localized hyperthermia, regardless of whether it is produced by radiofrequency, microwave radiation, ultrasound, or hot fluids, normal tissue tolerance will always be a limiting factor.

The effects of hyperthermia on a wide variety of animal and human tumors have been investigated, but relatively few studies have dealt with the effects of hyperthermia on normal tissues. Thus far reported have been the effects of hyperthermia on intestinal mucosa (3, 9), skin (15, 20), cartilage (3), bladder (2), bone marrow, and spinal cord (10). Watanabe (21) investigated the effect of applications of heat to the human scrotum and found that submerging the scrotum in water (43-47°) once daily for 30 min suppressed spermatogenesis and repeated heat treatments for 6 or 12 consecutive days reduced sperm counts to sterile levels. The purpose of this investigation was to quantitate the effects of moderate hyperthermia (41-44°), and hyperthermia combined with radiation, on spermatogenic stem cells using the spermatogenic colony assay (22), sperm head counts (7), testis weight loss (6), and fertility assays. The advantages and limitations of these assays have been discussed previously (7, 22).

MATERIALS AND METHODS

Animals. C57/Hf/Kam mice, maintained in a specific-pathogen-free colony, received treatments of heat and/or radiation when between 8 and 10 weeks of age. Mice were housed in groups of 5 in sterile mouse cages and fed sterile feed (Wayne Sterilizable Lab Block) and sterile acidified water (pH 2.5) ad libitum. The photoperiod was automatically regulated to a 12-hr light-dark cycle.

Irradiation Procedure. In preparation for radiation as well as heat treatments, sodium pentobarbital was injected i.p. at a dose of 54 mg/kg of body weight, ensuring both immobility of the animals as well as descent of their testes into the scrotal sacs. A pair of parallel-opposed 137Cs sources with a 3-cm diameter field was used for all irradiations (5). In order to keep the overall treatment time for all groups approximately equal, radiation doses of 575 rads or greater were delivered at a dose rate of 967 rads/min, while the dose rate was attenuated to 180 rads/min for doses less than 575 rads. The time interval between heating and irradiating never exceeded 5 min. For the combined modality groups, heat was always applied for 30 min prior to the radiation treatment.

Heating Procedure. Six mice, anesthetized as described earlier, were secured with tape to a specially designed Lucite mouse jig that allowed only the testes, hind legs, and tail to be immersed in the water bath. A Beckman thermocirculator was used to heat the water to the desired temperature. A mercury thermometer placed at the same depth as the testes was used to monitor the water temperature, and the readings were compared with that of a Bailey digital thermocouple thermometer (Model Bat 8) placed at the same depth. In addition, a needle thermocouple probe was inserted directly into the testis of a control mouse to monitor the actual temperature within the testis. The intratesticular temperature was independent of position on the mouse jig; i.e., the internal temperature of the testis was ±0.1° (S.E.) at each of the 6 mouse positions on the Lucite jig. The water temperature always read approximately 1.0° higher than did the temperature inside the testis. For example, a water temperature of 44° was needed to achieve a 43° internal testicular temperature. The normal testicular temperature of the anesthetized mice was 30°. Heat was always applied for a period of 30 min after the intratesticular temperature had been raised to the desired level (41-44°) over a 20-min period.

Spermatogenic Colony Assay. This assay measures the survival of spermatogenic stem cells (22). Briefly, mice were sacrificed and their testes were removed and submitted for routine histological processing...
35 days after heat and/or radiation. Sections were cut at 4 μm and stained with hematoxylin and eosin. A tubule was considered viable if at least 4 spermatogenic cells (not Sertoli cells) lay in close proximity to the tubule wall. Surviving fractions were calculated from the ratio of viable tubules to total tubules and the mean ± S.E. plotted as a function of radiation dose on semilogarithmic coordinates. Survival curves were drawn by fitting a line through the geometric mean values using a least-squares regression analysis and 95% confidence limits calculated for the D0 values describing the slopes of the lines.

**Sperm Head Assay.** With the method of Meistrich et al. (7), sperm head suspensions were prepared 56 days after treatment by homogenizing and sonicating the testis of each mouse in 2 ml of distilled water. Sperm heads were counted on a hemacytometer and the mean ± S.E. calculated for each treatment group.

**Testis Weight Loss.** Testis weight loss was measured 56 days after heat treatment. Testes were heated from 41–43.5° for 30 min. The mean ± S.E. of the testis weight in g was plotted as a function of temperature on semilogarithmic coordinates.

**Fertility Assay.** With the method of Meistrich (7), tests for fertility were carried out 56 days after hyperthermia (41–43° for 30 min). The treated male was housed with 3 virgin females. After 3 weeks, the females were checked daily for litter production. The time from heat treatment to fertility renewal was assumed to be 20 days (gestation period) prior to the date of the first litter.

**RESULTS**

**Mortality Associated with Hyperthermia.** The mortality associated with the heating procedure was approximately 1 to 2% at temperatures from 41–42.5°. The mortality at 43° was 22% (35 of 161), 44% (7 of 16) at 43.5°, and 88% at 44° (23 of 26). Death always occurred soon after the heating procedure and was assumed to be due to systemic heating of the mice, although rectal temperature was not monitored. Probibitive mortality was encountered when heating times exceeded 30 min.

**Testis Tubule Assay.** The curves shown in Chart 1 are for survival of spermatogenic stem cells assayed histologically (22) after radiation only or heat (41–43° for 30 min) plus radiation. The slope for the radiation alone survival curve can be described by a D0 value (radiation dose which reduces survival by one natural logarithm from 100 to 37%) of 174 rads while the 41° plus radiation curve is characterized by a D0 value of 136 rads. There is great variability in the stem cell survival data for testes heated to the higher temperature (42–43°). The D0 values and 95% confidence intervals are 67 (48 to 114) rads and 121 (74 to 330) rads for preheating to 42° and 43°, respectively. No particular significance can be attributed to the difference in slopes of the survival curves. The most important feature of these curves is their displacement to the left with increasing temperature. Other data to be presented indicate that this results mostly from independent cell killing by the heat; the contribution of cell sensitization by the hyperthermia cannot be accurately assessed from these survival data.

The effect of heating combined with radiation on spermatogenic stem cell survival is most reliably determined in the midrange of the survival curves (Chart 1). TER at a survival level of 0.1 are plotted in Chart 2. They are 1.27 at 41°, 1.80 at 42°, and 3.97 at 43°.

An asymmetrical distribution of surviving tubules in a cross-section of testis treated with heat was observed. This contrasts with the random distribution of surviving tubules observed after treatment with radiation only. This phenomenon has been reported previously by Merino et al. (9) who observed a higher survival of intestinal crypts in that segment of the gut adjacent to the mesenteric attachment as opposed to lower survival in the distal portion after heat treatment. A similar asymmetrical distribution of survivors was observed in the testes, although in this instance the area of highest survival forms a horseshoe-shaped distribution near the attachment of the main testicular blood vessel. This horseshoe-shaped distribution correlates with a cooling effect of blood flow to and from the testis. However, a second possibility is that the enhanced thermal effect on that portion of the testis furthest from the main blood vessel is related to its relatively poorer oxygenation and consequently increased pH gradient (4).

Another difference between the effects of heat and radiation is the destruction by heat of Sertoli cells and other stromal elements. A reduction in the number of Sertoli cells at temperatures of 42° and 43° was noted. In addition, tubules devoid of all spermatogenic cells except for numerous sperm in the
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30 41 43 43.5 41.5 42 42.6

Temp (°C)

Chart 3. A curve plotting absolute sperm head number (left ordinate) and surviving fraction (right ordinate) in testis homogenates 56 days after heating for 30 min to the temperatures shown on the abscissa. Points, mean. Bars, S.E.

- 0.01 £ 0.001 0.0001

100 300 500 700 900

Radiation DOM (Rad)

Chart 4. Sperm head counts obtained after testes were treated with 41, 42, and 43° plus varying doses of radiation. Testes were heated for 30 min immediately prior to irradiation, and sperm head counts were made 56 days after treatment. Absolute sperm head number and surviving fractions were plotted as a function of radiation dose on semilogarithmic coordinates. Points, mean. Bars, S.E.

- 0.01 £ 0.001 0.0001

100 300 500 700 900

Radiation (Rad)

Chart 5. Testes weight loss curve obtained from testes heated for 30 min to 42-43.5°. Testes weights were measured at 56 days after treatment and were plotted as a function of temperature on semilogarithmic coordinates. Points, mean; Bars, S.E.

Table 1

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Fertility</th>
<th>Sperm head production</th>
</tr>
</thead>
<tbody>
<tr>
<td>01°</td>
<td>41°</td>
<td>42°</td>
</tr>
<tr>
<td>10 mice</td>
<td>100%</td>
<td>70%</td>
</tr>
<tr>
<td>10 days</td>
<td>58%</td>
<td>70%</td>
</tr>
<tr>
<td>180 days</td>
<td>57-92</td>
<td>79-153</td>
</tr>
<tr>
<td>Median</td>
<td>1.56 ± 0.27</td>
<td>2.09 ± 0.15</td>
</tr>
<tr>
<td>Range</td>
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<td>4.45 ± 0.24</td>
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<td>% of control sperm head count</td>
<td>100</td>
<td>44</td>
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</table>

*a Mice mated 56 days after heating.

** Mean ± S.E. 

lumen were frequently seen in the heated groups. No reduction in the number of Sertoli cells was seen in radiation only groups, and it was rare to see a tubule containing sperm without also having present spermatogenic cells in other stages of differentiation. It seems probable that heat damage to the nonspermatogenic supporting cells resulted in sperm being unable to migrate normally out of the testis. This observation is supported by data obtained in the sperm head assay.

Sperm Head Assay. Due to the precise sequence of events which govern spermatogenesis, it has been possible to determine a time course of maturation for specific cell types throughout this process (8, 11–13). At 56 days after treatment, sperm head number is a direct reflection of spermatogenic supporting cells resulted in sperm being unable to migrate normally out of the testis. This observation is supported by data obtained in the sperm head assay.

The results of the 56-day sperm head assay after heat treatment alone are presented in Chart 3. At 41°, the sperm head number is reduced to a surviving fraction of 0.58 with a steady decline to a surviving fraction of 0.003 at 42.5° after which it plateaus. This plateau in the survival curve is attributed to the destruction of nonspermatogenic cells, particularly Sertoli cells, with the result that sperms heads already differentiated from stem cells 56 days before were unable to migrate out of the testis. Setchell et al. (17) have demonstrated previously that heat causes a decrease in fluid secretion by Sertoli cells, which is the principal means of transport for the spermatocytes out of the seminiferous tubules and from the testis to the epididymis (18).

Chart 4 shows the results obtained 56 days after the testis had been treated with heat plus radiation. The 41°-plus radiation curve is qualitatively similar to the biphasic radiation-only curve. Its origin is displaced downward by a factor of 2, reflecting the cytotoxic effect of the heat. There is further divergence of the survival curves as the radiation dose is increased, consistent with a radiosensitizing effect of the hy-
perthermia. The 42°C-plus radiation curve shows a downward displacement at its origin by a factor of 58 and is also biphasic. There is a suggestion of a radiosensitizing effect but survival is too low for accurate definition of the response at doses greater than 600 rads. The origin of the 43°C plus radiation curve is displaced downward from the normal unirradiated control by a factor of 175 with a further 4-fold decrease in sperm head displaced downward from the normal unirradiated control by a factor of 58 and is also biphasic. There is no further decrease in weight with increasing temperature, the reduced shoulders may be attributed to cell sensitization by hyperthermia.

**Testis Weight Loss.** Testis weight loss 56 days after heat treatment is plotted as a function of temperature in Chart 5. The curve is qualitatively similar to that for 56-day sperm head data shown in Chart 3. After heating to 41°C for 30 min, there is a reduction in testis weight from control by a factor of 1.08. Similar heating to 42.5°C reduces the testis weight by a factor of 4. There is no further decrease in weight with increasing temperature, presumably reflecting the several hundred-fold decrease in spermatogenic cell population (Chart 3), the residual weight being that of nonspermatogenic elements.

**Fertility Renewal Assay.** In an effort to quantitate the functional ability of those spermatozoa produced from stem cells that survived hyperthermia alone, male mice were mated with 3 virgin females 56 days after treatment. The results of the fertility assay 180 days after treatment are presented in Table 1 and show that the percentage of animals regaining fertility decreased as a function of increasing temperature. Ninety percentage of the mice whose testes were heated for 30 min at 41°C regained fertility. After similar heating to 42 and 43°C, the incidences of regained fertility were 70 and 40%, respectively. It can also be seen that the median length of time necessary to regain fertility increased with increasing temperature from 58 days at 41°C to 109 days at 42°C, and 142 days at 43°C.

Immediately after the first litter was recorded, the male was sacrificed and sperm head counts made in order to determine the number of sperm necessary for resumption of fertility. These results (Table 1) indicate that the minimum level of sperm necessary for resumption of fertility is 13% of the sperm head count of a normal mouse. Meistrich et al. (7) have shown that fertility is restored when the sperm head level reaches approximately 15% of control after 600 to 900 rads, although other investigators have reported that a level of 10% of control was sufficient after a 200-rad dose of radiation (16).

**DISCUSSION**

It is apparent from the data obtained from multiple assays of stem cell survival (spermatogenic colony regrowth, sperm head counts, testis weight loss, and fertility) that the testes are very sensitive to moderate degrees of hyperthermia. Although thermal enhancement ratios of several different normal tissues have been reported by other investigators (3, 9, 10, 15), a legitimate comparison between those TERs and the TER obtained in these experiments cannot be made because of the different levels of cell survival represented by the end points assayed, with cell survival being influenced by temperature, duration of heating, and the sequence of heating and irradiating. However, some idea of the sensitivity of testes to hyperthermia and radiation when compared with other normal tissues can be obtained by referring to Table 2. At 41 and 42°C, the TER calculated for spermatogenic cells are slightly greater than the TER for intestinal crypt cells; while at 43°C, it is substantially higher. Bone marrow cells appear to be even more sensitive to heat than are the testis stem cells, while the spinal cord would appear to be the least sensitive. Since different end points reflect different levels of cell survival, simple direct comparisons of TER obtained with different assays are unjustified. However, it is equally tenuous to claim, particularly for temperatures higher than 42°C, that TER probably do not vary much from tissue to tissue (3).

**ACKNOWLEDGMENTS**

We wish to thank Dr. Lester Peters for helping design the hyperthermia and radiation equipment and Dr. Marvin Meistrich for helpful discussions on the interpretation of sperm head data.

**REFERENCES**


Table 2

<table>
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<tr>
<th>Author</th>
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<th>TER</th>
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<tr>
<td>Merino et al. (9)</td>
<td>Intestine</td>
<td>Isoeffect 20 crypts/circumference</td>
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<td>Skin reaction</td>
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<td>1.18</td>
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<td>41</td>
<td>1.27</td>
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<td>Miller et al. (10)</td>
<td>Spinal cord</td>
<td>Paraplegia in 50% of animals</td>
<td>42</td>
<td>1.12</td>
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<td>Field et al. (3)</td>
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<td>LD₅₀</td>
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<td>1.90</td>
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<td>Skin reaction</td>
<td>43</td>
<td>2.06</td>
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<tr>
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<td>Testis</td>
<td>0.1 stem cell survival</td>
<td>43</td>
<td>3.97</td>
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<tr>
<td>Robinson*</td>
<td>Bone marrow</td>
<td>Ratio of survival curve slopes</td>
<td>43</td>
<td>4.39</td>
</tr>
</tbody>
</table>

* The abbreviation used: TER, thermal enhancement ratios.
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Effects of Hyperthermia and Radiation on Mouse Testis Stem Cells

Betty O. Reid, Kathryn A. Mason, H. Rodney Withers, et al.


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