Effects of microgravity or simulated launch on testicular function in rats

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AMANN, R. P., D. R. DEAVER, B. R. ZIRKIN, G. S. GRILLS, W. J. SAPP, D. N. R. VEERAMACHANENI, J. W. CLEMENS, S. D. BANERJEE, J. FOLMER, C. M. GRUPPI, D. J. WOLGEMUTH, AND C. S. WILLIAMS. *Effects of microgravity or simulated launch on testicular function in rats.* J. Appl. Physiol. 73(2), Suppl.: 174S–185S, 1992.—Testes from flight rats on COSMOS 2044 and simulated-launch, vivarium, or caudal-elevation control rats (5/group) were analyzed by subjective and quantitative methods. On the basis of observations of fixed tissue, it was evident that some rats had testicular abnormalities unassociated with treatment and probably existing when they were assigned randomly to the four treatment groups. Considering rats without preexisting abnormalities, diameter of seminiferous tubules and numbers of germ cells per tubule cross section were lower ($P < 0.05$) in flight than in simulated-launch or vivarium rats. However, ratios of germ cells to each other or to Sertoli cells and number of homogenization-resistant spermatids did not differ from values for simulated-launch or vivarium controls. Expression of testis-specific gene products was not greatly altered by flight. Furthermore, there was no evidence for production of stress-inducible transcripts of the *hsp70* or *hsp90* genes. Concentration of receptors for rat luteinizing hormone in testicular tissue and surface density of smooth endoplasmic reticulum in Leydig cells were similar in flight and simulated-launch rats. However, concentrations of testosterone in testicular tissue or peripheral blood plasma were reduced ($P < 0.05$) in flight rats to <20% of values for simulated-launch or vivarium controls. Thus spermatogenesis was essentially normal in flight rats, but production of testosterone was severely depressed. Exposure to microgravity for >2 wk might result in additional changes. Sequelae of reduced androgen production associated with microgravity on turnover of muscle and bone should be considered.

testis; spermatogenesis; intratesticular testosterone; Leydig cells; cellular-stress genes

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THERE IS seemingly contradictory evidence on effects of spaceflight or microgravity on testicular function in mammals. Although a slight decrease in number of spermatogonia per cross section of seminiferous tubule in testes from rats flown for 7 days on Spacelab 3 or 13 days on COSMOS 1887 were reported (23, 28), Serova et al.

(29) concluded that spermatogenesis was not affected in rats flown 7 or 13 days on COSMOS 1667 or COSMOS 1887. Serum concentrations of testosterone were low in rats after return from spaceflight (11), indicating that endocrine function of the testes might be altered by stress factors associated with spaceflight or effects of microgravity. The reasons for abnormalities of the endocrine and possibly the exocrine (spermatogenic) functions of the testes associated with spaceflight, at least in rats, are unknown but could be related to altered function of the hypothalamus or adenohypophysis (9), altered fluid distribution in the body (22), and/or restricted blood and lymph flow within the testis.

Starting with the assumption that stress factors associated with spaceflight, or effects of microgravity per se, represent simply a unique set of environmental toxins, we used approaches of reproductive toxicology and cellular and molecular biology to evaluate testicular function in rats from COSMOS 2044. Sensitive methods for quantification of alterations of testicular function by enumeration of germ cells, morphometric analyses, and techniques of cellular endocrinology have proven to be invaluable in identifying reproductive toxins (1–3, 17). As understanding of the genetic regulation of testicular function evolved, it became evident that eukaryotic organisms synthesize a subset of gene products in response to environmental stress; these have been termed cellular-stress or heat-shock proteins. Some members of this gene family also are expressed at specific points during spermatogenesis in mammals (33), and their expression is associated with specific germ cell types (34, 35). Thus expression of these genes provides molecular markers that can indicate normal spermatogenesis or can reveal whether a testis is responding to an altered environment (16).

METHODS

Animals and Tissue Collection

Details of the history, age, and body weight of the rats; assignment to four treatment groups (flight, simulated

launch, vivarium, and caudal elevation); animal care before and during the experiment; conditions of simulated or actual launch, flight, and reentry; and timing of tissue sampling relative to reentry are presented elsewhere (10). Briefly, flight rats were flown on an unmanned COSMOS satellite for 14 days, and tissues were sampled for analysis 11 h after return to earth. Simulated launch rats were subjected to vibrational and g-force stress but not microgravity. Additional control rats were subjected to caudal elevation (6, 13, 30) to unweight the hind limbs and simulate effects of thoracic pooling of body fluids. All rats were adult males of Czechoslovakian origin.

For each of five rats (rats 6–10) in the four groups, each testis was separated from its epididymis; both organs were trimmed free of extraneous tissue and weighed. The right testis was cut into three pieces; a central portion was placed into a container and immersed in liquid nitrogen (−196°C), and the two poles (each representing ~0.25 of the testicular length) were immersed into fixative at 4°C. The epididymis also was immersed in fixative. The fixative was Triple Fix (24), which contained 1% (wt/vol) paraformaldehyde, 3% (vol/vol) glutaraldehyde, 88 mM sucrose, and 2.5 mM difluorodinitrobenzene in 0.1 M sodium cacodylate buffer.

Frozen tissues remained in liquid nitrogen or on solid CO_2, and fixed tissues in 4°C fixative, until receipt at Colorado State University. There, frozen tissue was aliquoted, while at −79°C, for shipment at −79°C to Dr. Deaver for enumeration of homogenization-resistant spermatids and quantification of testosterone and luteinizing hormone receptors and to Dr. Wolgemuth for measurement of expression of cellular-stress genes. Fixed tissue also was divided: the poles of a testis were randomly designated as A or B, and the outer portion of each pole was sliced off and shipped to Dr. Zirkin in 0.1 M cacodylate buffer, at 4°C, for morphometric analysis of Leydig cells. The inner portion of each testicular pole was retained by Dr. Amann for subjective and objective evaluations of spermatogenesis and measurement of seminiferous tubule diameter; this tissue later was shared with Dr. Sapp. To enable deductions concerning onset of alterations in the seminiferous epithelium, we examined epididymal tissue microscopically to estimate the number of sloughed immature germ cells present among the spermatozoa.

Spermatogenesis

Histological evaluations. Thin slices of fixed tissue were rinsed in 0.1 M cacodylate buffer, immersed 1 h in OsO₄ [1% (wt/vol) in 0.1 M cacodylate buffer], rinsed in buffer, dehydrated through graded ethanols, and embedded in a mixture of Araldite and PolyBed-812 (1:1.64 by wt). For two to four blocks per testis pole, 4 × 6-mm sections were cut at 0.8 μm, collected on glass slides, and stained with toluidine blue. Each slide was coded with a number that precluded identification of treatment or end of the testis by observers making subjective or morphometric evaluations. Epididymal tissue was similarly processed. By the use of light microscopy, testicular tissue was subjectively evaluated, independently, by two individuals (RPA and DNRV) expert in spermatogenesis and pathophysiology, for normalcy of both the seminiferous epithelium and the

interstitial tissue. Diameters of 25 essentially round cross sections through seminiferous tubules and their lumina were measured for each of two to four loci (blocks) representing each testicular pole. For each stage VII cross section (18), the number of Sertoli cells with a discernible nucleolus, and numbers of nuclei of A spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, or step-7 spermatids were recorded. Diameters of two nucleoli of Sertoli cells and of two nuclei of each type of germ cell also were measured. Concurrently, for each tissue section, appearance of the seminiferous epithelium was classified as normal or having minor, moderate, or severe degenerative changes. Counts of germ cells were corrected for differences in nuclear diameter and section thickness using a modification of Abercrombie's formula (5), and the resulting data were used to calculate numbers of germ cells per Sertoli cell or ratios among germ cell types (2).

In addition, 2-μm sections were cut from selected blocks (abnormal rats were excluded) and stained with toluidine blue for enumeration of nuclei of spermatogonia (type A or B spermatogonia) and Sertoli cells in essentially round cross sections of stage VI seminiferous tubules. Tubules were staged on the basis of morphology of the spermatogonia (23), with verification of staging based on morphological features of spermatids (18). Nuclei in every other section through one, or occasionally two, stage VI tubule were counted until 50 cross sections had been scored. Counts were corrected for nuclear diameter and section thickness, after which the mean value was divided by 2.5 to make the data comparable to those for stage VII tubules (sections were 2.0 rather than 0.8 μm thick).

Efficiency of sperm production. The number of homogenization-resistant spermatids per gram of testicular tissue, a measure of the efficiency of sperm production (1), was determined by modifying the procedure of Robb et al. (27). Briefly, frozen tissue was thawed, weighed (131–253 mg), and minced with two razor blades. The tissue was transferred quantitatively with 2.0 ml buffer (0.5 M Tris-HCl, 0.23 M sucrose, 5 mM $MgCl_2$, and 4 mM NaN_3) into a 7-ml Dounce homogenizer and subjected to 10 strokes with pestle B followed by 10 strokes with pestle A. The homogenate was centrifuged (800 g for 10 min) at 4°C. The supernatant was saved (see Leydig Cell Function), and the pellet was resuspended in 2 ml buffer and homogenized a second time. After centrifugation, the supernatant was added to the previous one, and the pellet was resuspended in 5–10 ml of 0.145 M NaCl containing 4 mM NaN_3 and 0.05% (vol/vol) Triton X-100 (27). The number of homogenization-resistant (elongated) spermatid nuclei in each suspension was determined by cytometer counts; data were expressed on a tissue wet-weight basis. The resulting values should provide a sensitive measure of the efficiency of spermatogenesis; but because the homogenization procedure is different from that pioneered by Robb et al. (27), the absolute values are not comparable with those of others (2, 27).

Gene Expression

Molecular probes. Expression of hsp70 and hsp90 was examined. The hsp70 probe is a 1.3-kb HindIII-EcoRI

cDNA insert from plasmid pHMS213 (a gift from Dr. L. Moran; Ref. 18). This mouse cDNA recognizes high levels of a novel 2.7-kb transcript in spherical and elongating spermatids in normal mice (34, 35) and also heat-inducible 2.5- and 3.5-kb transcripts in heat-shocked L mouse cells. The *hsp90* probe is a 1.1-kb *PstI* cDNA insert from the plasmid pHS801 (a gift of Drs. E. Hickey and L. Weber; Ref. 15). This human cDNA recognizes a 3.2-kb transcript in mouse testis, expressed most abundantly in primary spermatocytes in prophase of the first division of meiosis (12), and also recognizes a 2.95-kb transcript present in heat-shocked or non-heat-shocked HeLa cells (15).

Analytic methods. Control testis and liver tissues were obtained from male rodents: Sprague-Dawley rats (Camm Research Industries), Swiss Webster mice (Camm Research Industries), and B6D2F1/J mice (Jackson Laboratory). Tissues were quickly plunged into liquid nitrogen and stored at −70°C. Total RNA was extracted from tissues by lithium chloride precipitation (4). Samples were processed in sets, with each set containing tissue from one rat in each of the four treatment groups from COSMOS 2044 plus samples of testis and liver from a normal rat and mouse as controls. Similar amounts of RNA from each sample were loaded onto denaturing gels (0.8% agarose, 2.2 M formaldehyde), electrophoresed, semi-dry electroblotted to Nytran membrane, and linked to the membrane by exposure to ultraviolet (UV) light for 5 min. Probes (see above) were labeled by random priming with [^{32}P]deoxynucleotide triphosphates (8). [^{32}P]probes were hybridized to mRNA on the filters at high stringency in the presence of dextran sulfate (31), washed sequentially for 20 min each in 2× standard saline citrate (SSC) plus 1% (vol/vol) sodium dodecylsulfate (SDS) at room temperature, twice in 2× SSC plus 0.1% SDS at 65°C, once in SSC plus 0.1% SDS at 65°C, once in 0.1× SSC plus 0.1% SDS at 65°C, and once in 0.1× SSC at 65°C (SSC = 0.15 M NaCl and 0.015 M sodium citrate). The integrity and relative abundance of RNA on the membranes was assessed by UV visualization of ethidium bromide incorporated by rRNA and recorded on Polaroid Type-55 film. Molecular weight of each mRNA transcript was estimated using the 28- and 18-S bands of rRNA as internal size standards. The 28-S rRNA is 4.729 kb in rats and 4.712 kb in mice, and the 18-S rRNA is 1.869 kb in both rats and mice (14, 26).

Hybridization was detected by exposure of a Northern blot to Kodak X-ray film, with an intensifying screen, at −70°C for 1–14 d. Both autoradiograms of Northern blots and negatives of gels were digitized (256-point gray scale) and relative amounts of RNA estimated by video image analysis with a JAVA system (Jandel Scientific). An arbitrary standard scale was used to calibrate intensity, with a randomly selected area of background set at 0 and a randomly selected area of high density assigned a value of 100. Densitometry values represented the average intensity over the entire area of a band. To provide a correction factor for unequal sample loading within a gel, the value for the most intense rRNA band in a gel was divided by the value for the corresponding rRNA in each sample. This correction factor for relative amount of total RNA loaded for each sample was used to adjust intensity values for the amount of mRNA or mRNA hybridization signal for that sample. Thus comparisons could be made among bands within a gel or Northern blot.

Leydig Cell Ultrastructure

For each testicular pole (A or B), 3–5 blocks of tissue were examined for each rat; thus 30–50 blocks of tissue were examined for the five rats in each treatment group. Thin sections from each block were mounted on formvar-coated, 200-mesh grids and stained with uranyl acetate and lead citrate. Grids were examined with a Hitachi HU-12A electron microscope. To minimize sampling bias, Leydig cells were initially selected at ×1,000 magnification, at which cytoplasmic organelles could not be visualized. Usually three micrographs, prepared at ×20,000 original magnification, were necessary to encompass each selected Leydig cell, and three Leydig cells on each grid were photographed. Thus 18–30 Leydig cells were examined per rat.

Prints were prepared (at ×2.75) so that all stereological determinations (7, 32, 37) were made on micrographs at a final magnification of ×55,000. Surface densities were determined for smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), outer mitochondrial membrane (OMM), and inner mitochondrial membrane (IMM). The 20 × 25-cm stereologic overlay was of a multipurpose test system, containing 112 short test lines (each terminating as a line-end point) arrayed as 14 rows of 8 test lines each. The overlay was placed over a micrograph and used to determine the surface density of each organelle (32) by counting intersections of test lines on the overlay with membranes of the organelles. Surface density (S_V) was calculated using the formula $S_V = 4(I)/(P_T)(d)$, in which I is the number of intersections between test lines and organelle membrane, P_T is the number of line-end points over cytoplasm of Leydig cells, and d is the length of the test line corrected for magnification. Repeatability was established by reanalysis, several months later, of 50 micrographs each from the vivarium and flight groups (including both normal and abnormal rats). For SER, OMM, and IMM, deviations from the first counts were 7, 4, and 8%. Thus it is probable that counting error was <10% for each organelle.

Leydig Cell Function

The pooled low-speed supernatants prepared from thawed testicular tissue (see *Spermatogenesis*) were centrifuged at 20,000 g for 15 min at 4°C. The supernatant was removed and testosterone concentration determined by radioimmunoassay. Reagents for the liquid-phase assay, using ^{125}I-testosterone-tyrosine methyl ester as the ligand, were from ICN (Costa Mesa, CA). All samples were quantified in a single assay and the intra-assay coefficient of variation was 11%. The assay was validated for rat testicular tissue by demonstrating that *1*) increasing amounts of tissue extract displaced ^{125}I-testosterone-TME in a dose-dependent manner parallel to the standard curve; *2*) tissue extracts did not bind detectable amounts of ^{125}I-testosterone-TME in the range of dilutions used to assay testicular testosterone; *3*) testoster-
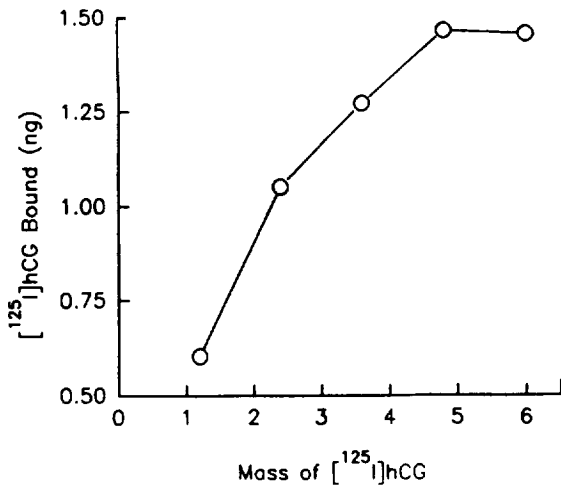
FIG. 1. Mass of human chorionic gonadotropin (hCG) required to saturate receptors for rat luteinizing hormone (rLH) in 20 mg rat testicular tissue in a total volume of 350 μl; incubated 18 h at 25°C. Data were corrected for nonspecific binding. See text for details.

one added to extracts was recovered in an amount essentially equal to that added; and 4) values obtained for aliquots of samples subjected to chromatography on Sephadex LH-20, to isolate testosterone from potentially cross-reacting compounds, were similar to values for nonchromatographic aliquots ($r = 0.95$; $n = 10$; slope = 1.1, and y-intercept = 0.05).

The capacity of membrane fragments in the 20,000 g pellet to bind human chorionic gonadotropin (hCG) was used to estimate the number of unoccupied receptors for rLH per gram of testicular tissue; presumably rLH receptors all were on Leydig cells. A "standard curve" technique was used, because there was insufficient tissue for a Scatchard analysis of each sample. A standard testicular membrane preparation was prepared, as above, using testes from 10 Sprague-Dawley rats 90–120 days old; it must be assumed that affinity of the rLH receptor is similar in American and Czechoslovakian rats. In developing the assay, the amount of hCG necessary to saturate rLH receptors in an aliquot of standard preparation of testicular membranes was determined. Duplicate aliquots representing 20 mg testicular tissue were incubated in 12 × 75-mm tubes with 1.2, 2.4, 3.6, 4.8, or 6.0 ng ^{125}I-hCG, prepared from NIDDK hCG CR-127 by the chloramine-T procedure, in a total of 350 μl buffer for 18 h at 25°C. Tubes were centrifuged 30 min at 9,000 g and the supernatant decanted; radioactivity of the pellet was determined. It was found (Fig. 1) that 4.8 ng ^{125}I-hCG was sufficient to saturate rLH receptors in an extract representing 20 mg testicular tissue. To further validate the assay, aliquots of 0, 5, 10, 15, or 20 mg of standard membrane preparation (in 100 μl buffer), 100 μl of ^{125}I-hCG solution containing 6.0 ng hCG, and 150 μl of buffer containing 0 or 100 IU (about a 1,000-fold excess of hCG; Sigma CG-10) were mixed and incubated for 18 h at 25°C. Tubes were centrifuged and radioactivity was measured as above; the amount of ^{125}I-hCG specifically bound was calculated as binding without excess ligand minus binding with excess ligand. Specific binding was directly proportional to the mass of testicular tissue present and increased in a linear manner from 0 to 20 mg

(Fig. 2). For analysis of the COSMOS 2044 samples, a series of duplicate aliquots of the standard membrane preparation representing 5–20 mg of tissue, or 20-mg aliquots representing each sample, were incubated with 6.0 ng ^{125}I-hCG plus 0 or 100 IU hCG, and specific binding quantified as above. All samples were included in one assay, and the intra-assay coefficient of variation was 4.8%.

Statistical Analyses

Data for organ weights, number of spermatogonia in stage VI seminiferous tubules, surface density of organelles in Leydig cells, concentration of testosterone in blood plasma (Ref. 21; data provided by R. E. Grindeland), and concentrations of testosterone or LH receptors were analyzed using the SAS General Linear Model (GLM) procedure and a one-way analysis of variance. Quantitative data for characteristics of seminiferous tubules and numbers or ratios of germ cells were analyzed using a two-way analysis of variance considering pole of the testis (2, random) and treatment group (3 or 4, fixed). All four treatment groups were included in analyses of diameters of seminiferous tubules and their lumina, but for other attributes the caudal-elevation rats were excluded because their seminiferous tubules contained virtually no germ cells. There was no significant difference between the A and B poles for any characteristic, except for corrected numbers of preleptotene spermatocytes (considered to be a type I error). If the F value associated with treatment was significant ($P < 0.05$), Duncan's multiple-range test was used to establish which groups differed ($P < 0.05$).

RESULTS AND DISCUSSION

General testicular function. Mean testicular weight for rats in the vivarium group did not differ from those for either the flight or simulated-launch groups, but testis weight was greater ($P < 0.05$) for rats in the simulated-launch group than those in the flight or caudal-elevation groups (Table 1). However, some rats in the flight and vivarium groups had small testes, and all rats in the cau-
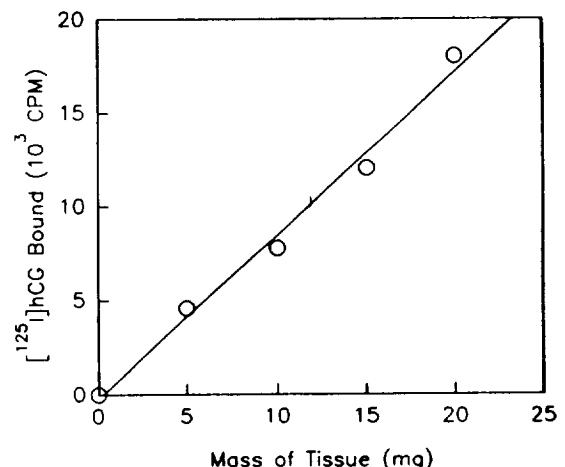

FIG. 2. Binding of ^{125}I-hCG to receptors for rLH as a function of mass of testicular tissue (0–20 mg/tube) incubated in 350 μl for 18 h at 25°C. Data were corrected for nonspecific binding. See text for details.

TABLE 1. *Characteristics of the right testis for all rats of COSMOS 2044 study*

	Flight	Simulated Launch	Vivarium	Caudal Elevation
Right testis wt, mg	960ᵃ	1,416ᵇ	1,048ᵃᵇ	492ᶜ
Right epididymis wt, mg	317ᵃ	401ᵃ	314ᵃ	242ᵃ
Seminiferous tubule diameter, μm	222ᵃ	266ᵇ	247ᵃᵇ	185ᶜ
Tubule lumen diameter, μm	90ᵃᵇ	109ᶜ	101ᵇᶜ	85ᵃ
Normal, %	49ᵃ	88ᵇ	65ᵃᵇ	nd
Early degeneration, %	7ᵃ	5ᵃ	5ᵃ	nd
Moderate degeneration, %	5ᵃ	5ᵃ	5ᵃ	nd
Severe degeneration, %	39ᵃ	3ᵇ	21ᵃᵇ	nd
Stage VII seminiferous tubules				
Nuclei/tubule cross section				
Sertoli cells	1.26ᵃ	2.15ᵇ	1.41ᵃ	nd
Type A spermatogonia	1.57ᵃ	0.88ᶜ	0.84ᵃ	nd
Preleptotene spermatocytes	4.40ᵃ	6.65ᵇ	5.25ᵃᵇ	nd
Pachytene spermatocytes	4.24ᵃ	7.15ᵃ	6.00ᵃ	nd
Spermatids	13.67ᵃ	21.09ᵇ	16.64ᵃᵇ	nd
Ratio (no./Sertoli cell)				
Type A spermatogonia	1.08ᵃ	0.42ᵃ	0.63ᵃ	nd
Preleptotene spermatocytes	3.46ᵃ	3.14ᵃ	3.85ᵃ	nd
Pachytene spermatocytes	3.40ᵃ	3.38ᵃ	4.23ᵃ	nd
Spermatids	10.82ᵃ	9.96ᵃ	12.14ᵃ	nd
Ratio				
Preleptotene-to-A spermatogenic	9.13ᵃ	7.71ᵃ	6.44ᵃ	nd
Pachytene-to-preleptotene	0.98ᵃ	1.08ᵃ	1.10ᵃ	nd
Spermatid-to-pachytene	3.22ᵃ	2.95ᵃ	3.08ᵃ	nd
Spermatids/g testis, $\times 10^6$	28.9ᵃ	37.8ᵃ	33.2ᵃ	<0.5ᵇ
LH receptors, ng hCG bound/g testis	65.8ᵃ	56.8ᵃ	63.1ᵃ	63.7ᵃ
Testosterone, ng/g testis	7.0ᵃ	15.1ᵃ	64.5ᵇ	23.4ᵃ
Testosterone,† ng/ml blood plasma	0.27ᵃ	1.91ᵇ	3.50ᶜ	0.51ᵃ
Leydig cells, $\mu m^2/\mu m^3$				
Smooth endoplasmic reticulum	7.75ᵃ	7.23ᵃ	6.07ᵇ	7.85ᵇ
Rough endoplasmic reticulum	0.59ᵃ	0.66ᵃ	1.06ᵇ	0.87ᵇᶜ
Outer mitochondrial membrane	1.43ᵃ	1.47ᵃ	1.71ᵇ	1.48ᵇ
Inner mitochondrial membrane	3.85ᵃ	3.76ᵃ	4.26ᵃ	3.94ᵇ

Means (n = 5) with the same superscript letter do not differ ($P > 0.05$). nd, not determined. † Data for testosterone in blood plasma are from Merrill et al. (21).

dal-elevation group had very small testes. The probable causes of these differences were revealed by subjective histological evaluations of each testis and attached epididymis (Fig. 3), and confirmed by quantitative data.

One must assume that vivarium rats had not been exposed to stress factors associated with launch or flight and were the best representation of the population of rats shortly before launch. However, in three vivarium rats, one or both testes weighed ≤900 mg, and histological examinations of three of these four testes (only right testes were available) revealed that they were abnormal (see below). Similarly, right testes from two flight rats weighed ≤650 mg and had histological abnormalities similar to those in abnormal vivarium rats but different from those characteristic of caudal-elevation rats. Thus the cause of lesions in the testes of these two abnormal flight rats probably was the same as that inducing lesions found in three vivarium rats and was not flight. By this reasoning, three flight rats (7, 9, and 10), five simulated-launch rats (6–10), and two vivarium rats (7 and 10) did not have marked preexisting abnormalities; weights of their right testes ranged from 1,200 to 1,500 mg, whereas the right testes for the other five rats in these three groups weighed 450–900 mg. Thus we concluded that testes of some rats in the flight and vivarium and possi-

bly the caudal-elevation groups had been abnormal when they were assigned to treatment groups. By random chance, all rats in the simulated-launch group had normal testes.

Lesions in testes of rats with a preexisting abnormality were different from those in caudal-elevation rats. On the basis of testicular weight, the lesion was unilateral in some rats with preexisting abnormalities; but for caudal-elevation rats the lesion inevitably was unilateral. For caudal-elevation rats, the entire testis was uniformly affected (based on histological examinations of both poles and data for efficiency of sperm production in the central portion of the testis). Seminiferous tubules contained only Sertoli cells plus a complement of spermatogonia, a few primary spermatocytes, and occasional spermatids (Fig. 3, A and B). In contrast, in an abnormal testis from a flight or vivarium rat, there was a mixture of normal and abnormal seminiferous tubules (Fig. 3, D and E); and the extent of involvement was not uniform throughout the entire testis. On the basis of histological evaluations, testes from rats with a preexisting abnormality should have been producing spermatozoa, although in reduced numbers. These subjective observations were confirmed by data for efficiency of sperm production in the central portion of each testis (<0.5 vs. 22 × 10⁶ sper-
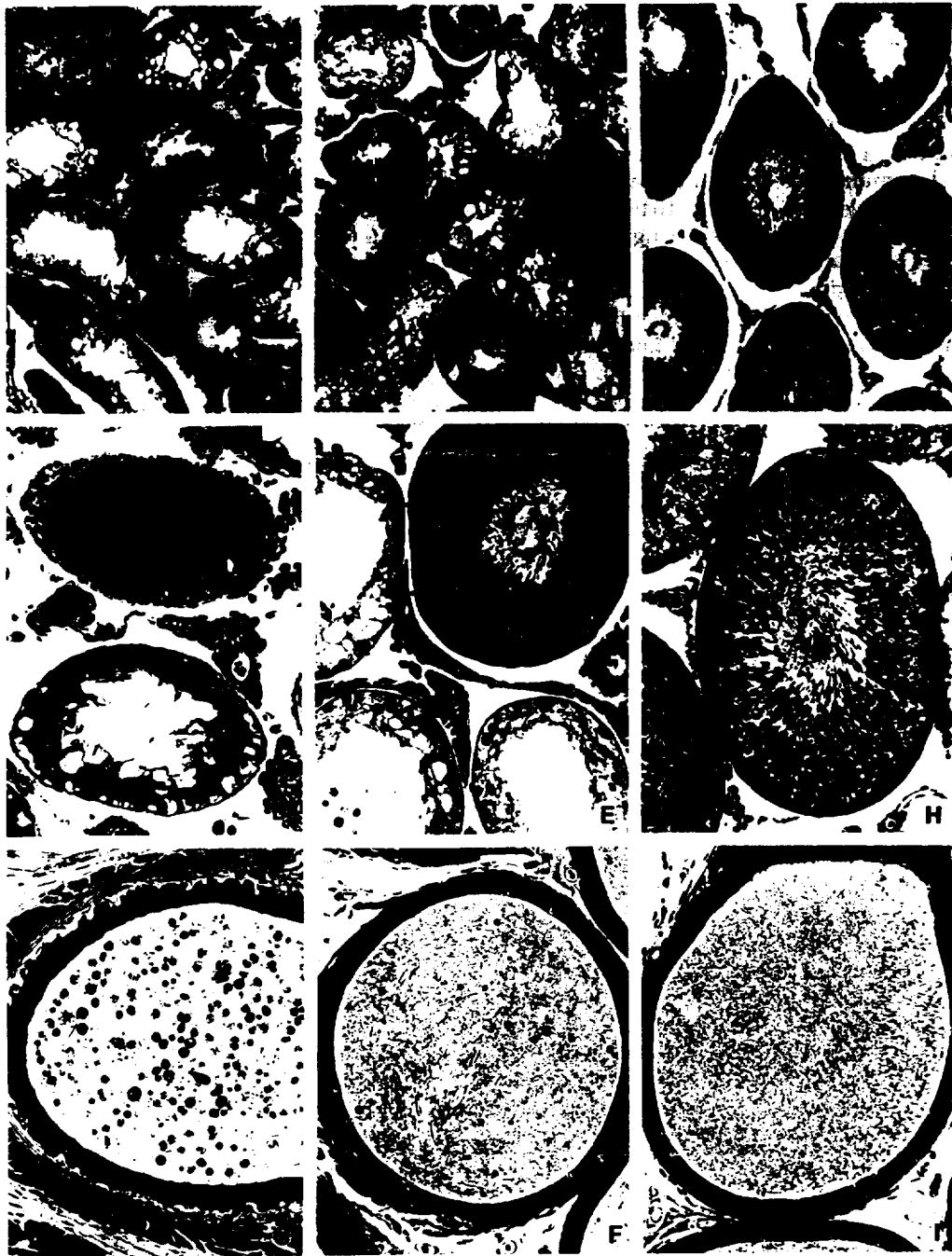
FIG. 3. Micrographs of testicular (A, B, D, E, G, and H) and epididymal (C, F, and I) tissue. Testicular tissue from a caudal-elevation rat (A and B) contained only abnormal seminiferous tubules, but a few germ cells were present in some tubules. These lesions were induced by treatment. Testicular tissue from a vivarium rat with a preexisting abnormality (D and E) contained both normal and abnormal seminiferous tubules; abnormal ones contained few germ cells and Sertoli cells predominated. Distinction of preexisting testicular abnormalities from those induced by caudal elevation also was evident from content of cauda epididymidis; in caudal-elevation rats sloughed germ cells rather than spermatozoa predominated (C), whereas in rats with preexisting lesions there were few sloughed germ cells among spermatozoa in cauda epididymidis (F). Testicular tissue from flight rats without a preexisting testicular abnormality was normal in appearance (G) and stage VII seminiferous tubules (H) had normal ratios of germ cells to Sertoli cells (see text and Table 2); luminal content of cauda epididymidis also was normal (I). A, D, and G are at ×62 and B, C, E, F, H, and I are at ×125 magnification; toluidine blue staining.

matids/g testis for caudal-elevation and preexisting abnormality rats). Other quantitative differences (P < 0.05) were evident. Caudal-elevation rats had a smaller right testis than rats with preexisting abnormalities, with smaller seminiferous tubules, and lower intratesticular and plasma concentrations of testosterone (492 vs. 690 mg; 185 vs. 228 μm diam; 23.4 vs. 60.9 ng/g; and 0.51 vs. 2.85 ng/ml) but a similar concentration of receptors for rLH in testicular tissue (63.7 vs. 78.8 ng/g). Morphometric characteristics of Leydig cells were similar for both

groups (data not presented). In caudal-elevation rats, the epididymis contained numerous germ cells sloughed from the seminiferous epithelium (Fig. 3C), which were indicative of recent destruction of the seminiferous epithelium. Undoubtedly the testes of caudal-elevation rats fell into the abdominal cavity (6, 29), through the inguinal canal which remains patent in rats (in contrast to humans), and were damaged by exposure to body temperature. Epididymides of rats with preexisting abnormalities had few immature germ cells among the spermatozoa (Fig. 3F), which precluded extensive destruction of the seminiferous epithelium within 2 wk before tissue fixation. In flight rats without preexisting abnormalities, spermatogenesis was normal (Fig. 3, G and H), and the cauda epididymidis was filled with spermatozoa (Fig. 3I).

The high values for intratesticular concentrations of testosterone and receptors for rLH in caudal-elevation rats and rats with preexisting testicular abnormalities probably reflect atrophy of the seminiferous tubules so that Leydig cells constitute a larger proportion of the testis. However, it is possible that these rats had more Leydig cells or Leydig cells that secreted more testosterone.

Only tissues from rats 6-10 in each of the four treatment groups were available to us for detailed examination. However, it is likely that at least two of the other rats (F-3 and V-1) had abnormal testes before assignment to the project, based on testicular weight (≤850 vs. 1,200-1,650 mg). Thus at least 7 of 30 rats (23%) in the flight, simulated-launch, and vivarium groups probably were abnormal before imposition of treatments involved in the COSMOS 2044 study.

Spermatogenesis. Considering the facts that testes of several rats probably were abnormal before assignment to treatment, and that groups of five rats/group are of marginal size to detect subtle treatment effects (3), dogmatic conclusions concerning effects on spermatogenesis of exposure to the stress factors of simulated or actual launch and reentry, or exposure to microgravity per se, are impossible. Table 2 summarizes the data after exclusion of rats presumed to have preexisting testicular abnormalities and is considered the best basis for conclusions concerning effects of flight. However, data for all rats are presented in Table 1 because minor sequelae on spermatogenesis of the unknown agent or genetic factor causing preexisting abnormalities in some rats may have been undetected in others. Considering all rats, based on subjective evaluations (Fig. 3) and quantitative data (Table 1), spermatogenesis in flight rats generally was similar to that in vivarium rats, but inferior to that in simulated-launch rats. This probably reflects the variable impact of preexisting abnormalities in certain rats. If there had been a profound treatment effect, the corrected numbers of germ cells per stage VII seminiferous tubule cross section, or one or more of the ratios between a specific type of germ cell and Sertoli cells, would have been reduced. Although such differences were detected between the flight and simulated-launch groups for preleptotene spermatocytes and step-7 spermatids, there was no difference for the ratios. Because the ratio of preleptotene spermatocytes to type A spermatogonia was not reduced, spermatogonia were completing mitosis. Ratios of pa-

TABLE 2. *Characteristics of the right testis in rats without preexisting abnormalities in the flight, simulated-launch, and vivarium groups of COSMOS 2044 study*

	Flight	Simulated Launch	Vivarium
n	5	5	2
Right testis weight	1230^a	1420^b	1450^b
Seminiferous tubule diameter, μm	229^a	260^b	267^b
Tubule lumen diameter, μm	96^a	106^a	106^a
Stage VI seminiferous tubules			
Total spermatogonia	5.26^a	5.57^{ab}	5.79^b
Stage VII seminiferous tubules			
Nuclei/tubule cross section			
Sertoli cells	1.04^a	2.15^b	1.70^{ab}
Type A spermatogonia	0.38^a	0.88^b	0.89^b
Preleptotene spermatocytes	3.65^a	6.65^b	6.10^b
Pachytene spermatocytes	3.92^a	7.15^b	7.20^b
Spermatids	11.93^a	21.09^b	20.57^b
Ratio (no./Sertoli cell)			
Type A spermatogonia	0.35^a	0.42^a	0.51^a
Preleptotene spermatocytes	3.44^a	3.14^a	3.69^a
Pachytene spermatocytes	3.67^a	3.38^a	4.24^a
Spermatids	11.18^a	9.96^a	12.42^a
Ratio			
Preleptotene-to-A spermatogenic	10.98^a	7.71^a	7.43^a
Pachytene-to-preleptotene	1.06^a	1.07^a	1.17^a
Spermatid-to-pachytene	3.08^a	2.95^a	2.92^a
Spermatids/g testis, 10^6	42.3^a	37.8^a	38.2^a
LH receptors, ng hCG bound/g testis	55.8^a	56.8^a	44.0^a
Testosterone, ng/testis	3.81^a	15.06^b	20.82^b
Testosterone,† ng/ml blood plasma	0.23^a	1.91^b	1.96^b
Leydig cells, μm^2/μm^3			
Smooth endoplasmic reticulum	7.93^a	7.23^a	5.51^b
Rough endoplasmic reticulum	0.53^a	0.66^a	1.05^a
Outer mitochondrial membrane	1.46^a	1.47^a	1.64^a
Inner mitochondrial membrane	3.98^a	3.76^a	4.08^a

Rats with a testis ≤0.90 g and abnormal histological appearance were considered to have a preexisting abnormality. Data for corrected number of spermatogonia in 2.0-μm sections for stage VI were divided by 2.5 to make them comparable to those for 0.8-μm sections for stage VII. Means with the same superscript letter do not differ ($P > 0.05$). † Data for testosterone in blood plasma are from Merrill et al. (21).

chytene spermatocytes to preleptotene spermatocytes and step-7 spermatids to pachytene spermatocytes were similar, so primary spermatocytes were completing meiosis. Thus the differences detected may not reflect a treatment effect.

At the penalty of reducing the number of available animals, data also were summarized after excluding the two flight and three vivarium rats with obvious preexisting abnormalities in the right testis (Table 2). The reductions ($P < 0.05$) in weight of the right testis and seminiferous tubule diameter persisted in flight rats, as did differences in numbers of Sertoli cells or germ cells per seminiferous tubule cross section. However, as for the complete data set, the ratios of germ cells to Sertoli cells or among types of germ cells were not affected by treatment. The number of homogenization-resistant spermatids per gram of testis was not affected by treatment and was independent of testicular weight ($r = -0.34$; $P > 0.05$).

Counts of all spermatogonia in cross sections of stage VI tubules (by CSW) were consistent with those for type

A spermatogonia in stage VII (by *DNRV*). In stage VI cross sections, there were fewer ($P < 0.05$) spermatogonia in testes from normal flight rats than in testes of simulated-launch or normal vivarium rats (Table 2), although the number of Sertoli cell nuclei was not significantly different (data not shown). The 5.3% reduction in number of total spermatogonia per cross section of stage VI seminiferous tubule for flight rats, relative to simulated-launch rats, was similar to results of Sapp et al. (28) for rats flown on Spacelab 3 or COSMOS 1887.

Diameter of seminiferous tubules in normal flight rats was less than in normal simulated-launch or vivarium animals (Table 2), despite apparent normalcy of spermatogenesis. This might reflect a distortion of the seminiferous tubules induced by microgravity, which persisted through the 11 h between landing and fixation of tissue. Although there is no known precedent, it is possible that there was a decrease in diameter and an offsetting increase in length of seminiferous tubules (quality of the material precluded determination of percentage seminiferous tubules, and hence tubule length), so that internal surface area of the basement membrane and space available for each Sertoli cell were essentially unaltered. Such changes would explain the decreases in numbers of Sertoli cells and germ cells per seminiferous tubule cross section, whereas the ratios of germ cells were normal and number of homogenization-resistant spermatids per gram of testis was unaffected.

Given the totality of the qualitative and quantitative findings (Tables 1 and 2), it is unlikely that exposure to microgravity for ≤ 2 wk had an immediate deleterious effect on mitosis of spermatogonia, survival of spermatocytes through meiosis, or maturation of spermatids. If the Sertoli cells or germ cells were affected directly by microgravity, or indirectly by reduced secretion of testosterone, other hormones, or regulatory factors, the lesion(s) must be either subtle or not discernible after 14 days of flight.

Gene expression. Yield of total RNA ranged from 39 to 600 μg and probably was a function of the mass of testicular tissue available. The weight of each sample of testicular tissue was not determined, because thawing the tissue before extraction might have resulted in degradation of the RNA. Efforts were made to load similar amounts of RNA (20 μg) on each lane of the gels used for Northern analyses, and data later were normalized within each gel to minimize the effect of differences in the amount of RNA loaded. Figures 4 and 5 show, respectively, samples of RNA from rats without (Fig. 4) or with (Fig. 5) preexisting abnormalities of the right testis.

The expression of the *hsp* genes was given high priority in this study because several members of the *hsp*70 and *hsp*90 gene families are expressed in specific germ cells (see METHODS). Thus they can serve as molecular markers of the presence of these germ cells. Moreover, certain members of the *hsp*70 and *hsp*90 gene families respond to cellular stress and are affected by altered testicular environment, including elevated temperature. A stress response in testicular cells could be elicited by a rise in temperature induced by movement of the testes from the scrotum into the body cavity during exposure to microgravity or caudal elevation or could be elicited by

one or a combination of other factors associated with flight. Availability of testicular samples from caudal-elevation rats, which became cryptorchid, enabled distinction of heat effects from possible effects induced by other aspects of spaceflight.

From the data depicted (Figs. 4 and 5), and similar data for the other four sets of tissue, two conclusions were reached. First, effects on levels of testis-specific gene products *hsp*70 and *hsp*90 reflected the preexisting abnormalities present in some rats, where there was no effect of spaceflight on normal expression of testis-specific *hsp* gene products. Flight, vivarium, and simulated-launch rats without preexisting testicular abnormalities (Fig. 4, lanes 1–3) had more of the germ-cell-specific 2.7-kb *hsp*70 and 3.2-kb *hsp*90 transcripts (mRNA) than flight or vivarium rats with preexisting abnormalities (Fig. 5, lanes 1 and 2) or caudal-elevation rats (Fig. 4, lane 4, and Fig. 5, lane 3). Reductions of *hsp*70 and *hsp*90 transcripts in certain flight and vivarium rats reflected corresponding preexisting abnormalities in the seminiferous epithelium (Fig. 3), including a reduction in the proportion of germ cells to somatic cells within the testis. The pronounced reduction in amount of germ-cell-specific mRNA in caudal-elevation rats, in comparison to rats with preexisting lesions, supports the morphological distinction (see above) between preexisting abnormalities and those induced by caudal elevation. Second, we observed no induction of the heat- or stress-inducible *hsp*70 transcripts of 2.5 and 3.5 kb or *hsp*90 transcript of 2.95 kb in any sample. Thus neither spaceflight nor caudal elevation for ≤ 2 wk induced detectable transcription of the heat-inducible *hsp*70 or *hsp*90 members of the cellular stress protein gene family. It may be productive to examine patterns of cellular stress-protein gene expression after several days of caudal-elevation, while the heat-induced lesion is forming but before substantial sloughing of the seminiferous epithelium. Furthermore, the possible induction of a cellular stress in the testes response to long-duration spaceflight remains to be assessed.

Leydig cell ultrastructure. In terms of surface density, Leydig cells in vivarium rats contained less ($P < 0.05$) SER and more ($P < 0.05$) RER and OMM than Leydig cells in rats from the other three groups (Table 1). Surface density of IMM did not differ among treatment groups.

When data for the caudal-elevation and rats with preexisting abnormalities were deleted, the only significant difference remaining among treatment groups was that for SER (Table 2). Surface density for this component was higher in flight or simulated-launch rats than in vivarium rats. Surface density of SER averaged 5.5 μm^2/ μm^3 in Leydig cells of vivarium rats, which is consistent with published data (37) for normal rats.

Leydig cell function. The number of unoccupied receptors for rLH in the testis, as estimated by binding of hCG, was similar for all four treatment groups (Table 1). Nevertheless, it was evident that Leydig cells in flight and simulated-launch rats produced much less testosterone than those in vivarium rats. This was reflected in both concentration of testosterone in whole testicular tissue and in plasma from trunk blood (Table 1). However, these conclusions probably were influenced by inclusion
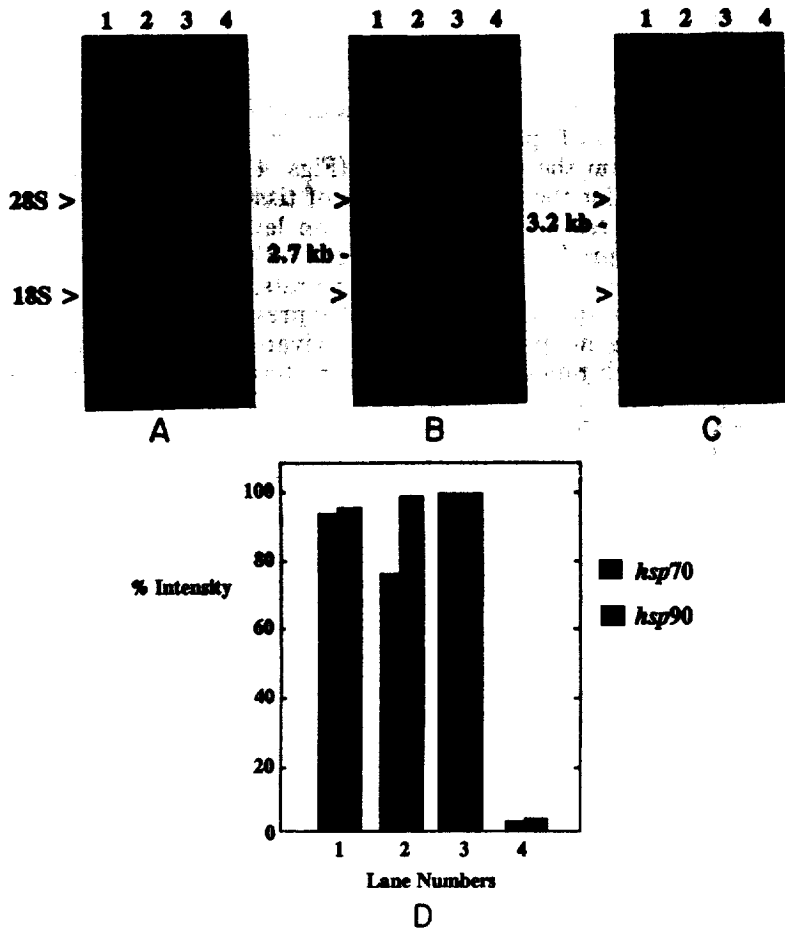
FIG. 4. Expression of cellular stress-protein genes in testes of rats. Total RNA was isolated from testes of rats 7, which had been subjected to flight (lane 1), vivarium (lane 2), simulated launch (lane 3), or caudal elevation (lane 4). Except for caudal-elevation rat, in which induced lesion precluded discernment of any preexisting abnormality, rats were normal before treatment. Total RNA was size fractionated by gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet (UV) light (A). The 28- and 18-S rRNAs are designated. Hybridization of the ^{32}P-labeled hsp70 (B) and hsp90 (C) probes to mRNA after transfer to membranes was detected by autoradiography. Relative amount of hybridization (D) was determined by densitometric analysis.

of rats with preexisting abnormal testes. Considering only the normal rats, mean concentrations of testosterone in testicular tissue or blood plasma both were lower in flight rats than in either simulated-launch or vivarium rats (Table 2). Thus it appears that testosterone production was reduced in flight rats that had been normal before the experiment but was similar in simulated-launch and vivarium rats without preexisting testicular abnormalities. Values for concentration of receptors for rLH were similar for all three of these groups (Table 2).

On the basis of data for 10 normal rats in the flight, simulated-launch, and vivarium groups, the correlation between intratesticular concentrations of testosterone and receptors for rLH ($r = 0.19$) was nonsignificant, although that between concentrations of testosterone in testicular tissue and in blood plasma was significant ($r = 0.66$). Surprisingly, the correlation between intratesticular concentration of testosterone and surface density of smooth endoplasmic reticulum was negative ($r = -0.62$; $P = 0.055$), although typically the surface area of SER or peroxisomes is highly and positively correlated with testosterone secreting capacity (7, 20, 36, 37).

Surface density of organelles (surface area per unit volume of Leydig cell) was determined in this study, rather than surface area per Leydig cell or per testis (7). This was done because differential shrinkage of tissue and artifacts associated with imperfectly fixed tissue precluded enumeration of Leydig cells per testis. Leydig cells used to determine surface density of organelles were

properly fixed, but rejection of some cells might have resulted in selection of an atypical sample. However, failure to find a significant, positive correlation between surface density of SER and intratesticular testosterone concentration probably is not a consequence of having measured surface densities rather than surface area. Surface density is highly correlated with the capacity of the testis to produce testosterone under conditions of maximal LH stimulation (36, 37). More plausible explanations for our observations are that 1) intratesticular testosterone at any instant need not reflect the capacity of a testis to secrete testosterone, 2) testosterone production in flight rats was considerably less than would be expected given the concentration of rLH receptors in the tissue and the surface density of SER, 3) Leydig cells might have undergone death and degeneration, or 4) we obtained a spurious result associated with the interval between landing and tissue sampling. No report of instant-to-instant changes in intratesticular concentration, in relation to surface density of SER, has been published. Although data for the concentration of LH in blood plasma from these rats are not available, we speculate that the problem probably is one of intratesticular fluid dynamics or changes in availability of oxygen and substrates to Leydig cells.

GENERAL DISCUSSION

Utility of the caudal-elevation model. The caudal-elevation (tail suspension or hind limbs unweighted) model
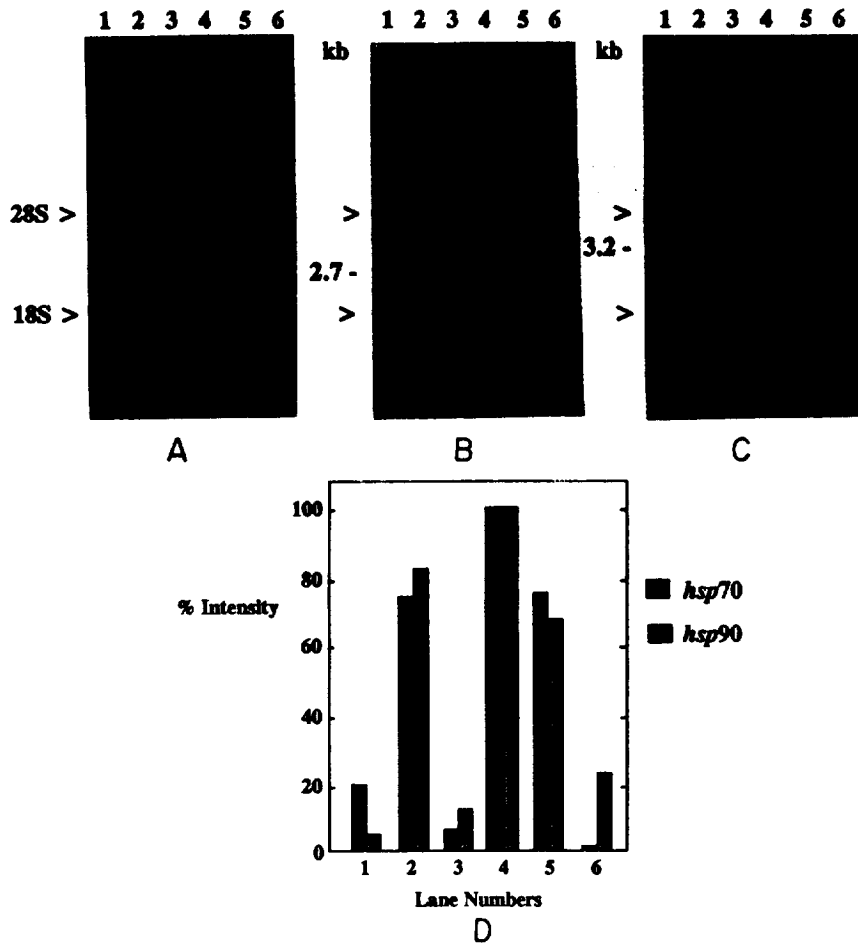
FIG. 5. Expression of cellular stress-protein genes in rats with or without preexisting testicular abnormalities. Total RNA was isolated from testes of rats 6, which had been subjected to flight (lane 1), vivarium (lane 2), caudal elevation (lane 3), or simulated launch (lane 4); control RNA was from normal mouse testis (lane 5; positive control) and mouse liver (lane 6; negative control). Testes of flight and vivarium rats had preexisting abnormalities, whereas simulated-launch rat was normal before treatment. Total RNA was size fractionated by gel electrophoresis, stained with ethidium bromide, and photographed under UV light (A). The 28- and 18-S rRNAs are designated. Hybridization of ^{32}P-labeled hsp70 (B) and hsp90 (C) probes to mRNA after transfer to membranes was detected by autoradiography. The hsp90 probe cross-hybridized with the 28- and 18-S rRNA. Relative amount of hybridization (D) was determined by densitometric analysis.

frequently is used to simulate effects of microgravity to study atrophy of muscle or loss of calcium from bone after elimination of their need to support the animal, but it also results in tissue fluid shifts (13). The present data confirm earlier data (6, 29), in that both spermatogenesis and testosterone secretion are abnormal in caudal-elevation rats. Two separate problems exist in rats in which caudal-elevation is achieved by tail suspension: 1) the testes descend into and remain in the abdominal cavity, and the resulting rise in intratesticular temperature causes destruction of the germinal epithelium; and 2) positioning the testes at a level equal to or above the heart alters blood flow to the testis and intratesticular fluid dynamics (probably affecting balance among blood, interstitial fluid, lymph, and seminiferous tubule fluid) and reduces secretion of testosterone.

Separation of these two sources of damage, by studying caudal-elevation rats in which the testes were retained in the scrotum by a loose ligature around the inguinal canal before suspension with a tail sling or alternatively (without surgery) by use of a whole-body harness (6), revealed that concentrations of testosterone in testicular interstitial fluid and in blood serum were abnormally low even in caudal-elevation rats with intrascrotal testes and normal spermatogenesis. Maintenance of normal spermatogenesis in caudal-elevation rats with intrascrotal testes, despite low testosterone concentrations in interstitial fluid of ∼125 ng/ml (compared with 350 ng/ml for control rats; Ref. 9), is possible because spermato-

genesis proceeds normally if testosterone is present in testicular tissue at 20–25% of the normal intratesticular concentration (38). Thus, in caudal-elevation rats from the COSMOS 2044 project, collapse of spermatogenesis was primarily a consequence of elevated temperature, whereas the decreased blood plasma concentration of testosterone (0.5 vs. 1.9 ng/ml for normal rats) was a consequence of altered fluid flow to and within the testis. As noted above, the measured intratesticular concentration of testosterone probably remained near normal because the proportion of the testis occupied by seminiferous tubules was greatly reduced.

Regardless of whether a whole-body harness or a tail sling is used to provide caudal elevation, testosterone production is decreased (6). However, the reduction of testosterone concentration in peripheral blood plasma of caudal-elevation rats was less than that for normal flight rats in the COSMOS 2044 study (0.51 vs. 0.23 ng/ml; 1.93 ng/ml in normal simulated-launch or vivarium rats). Thus the caudal-elevation model should not be rejected because testosterone production is reduced. Although the suppression of testosterone in caudal-elevation rats imperfectly mimics what may happen in microgravity, testosterone production is reduced in flight, so some sequelae of androgen deprivation might be detected using the caudal-elevation model.

Effects of microgravity on the testis. In flight rats from COSMOS 2044 without preexisting lesions, spermatogenesis was normal, based on subjective evaluations and

most quantitative data, but testosterone concentrations in testicular tissue and blood plasma were low. However, in the normal flight rats, the testicular testosterone concentration of 3.8 ng/g whole tissue was about twice that in peripheral blood plasma for the seven normal rats in the simulated-launch and vivarium groups (1.9 ng/ml). It is likely that testosterone production in normal flight rats was very close to the minimal amount obtained for normal spermatogenesis (20–25% of the normal testicular concentration; Ref. 38). If fluid distribution in the body or among the testicular compartments were further altered in flights of >13 days, further suppression of testosterone production probably would occur; and abnormalities or cessation of spermatogenesis would follow. We speculate that testosterone production in rats exposed to microgravity for ≤14 days, in COSMOS or similar flights, is barely adequate to maintain spermatogenesis. In flights of longer duration, spermatogenesis might be altered or further shifts in endocrine homeostasis might occur.

Decreased secretion of testosterone (and probably other gonadal steroids including estradiol) is reflected in lower concentrations in peripheral blood from flight rats (Ref. 11; Tables 1 and 2). This would affect not only the hypothalamus and adenohypophysis but also anabolism and catabolism of protein in muscle and deposition and retention of calcium in bone. It would seem prudent for individuals interested in nonreproductive aspects of exposure of mammals to microgravity to consider altered hormonal concentrations as a contributory proximate cause of any change detected in muscle, bone, or other body systems. Beneficial effects of prophylactic steroid therapy on maintenance of muscle mass or bone density of mammals exposed to microgravity should be considered, because such therapy is beneficial in reducing muscle changes in caudal-elevation rats (30).

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