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Effects of a Simulated Microgravity Model on Cell Structure and Function in Mouse Testis

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Abstract

Decreased testicular blood flow as a consequence cephalad fluid shift occurring under microgravity conditions causes impaired spermatogenesis in males. The tail suspended model has been used mainly in studies of muscle atrophy/osteoporosis and of body fluid shift in few studies aimed at spermatogenesis. We examined serum testosterone levels, testis morphology and apoptotic phenomena in tail suspended mice, a model used to reproduce some of the effects of the absence of gravity. Male C57BL mice (6 months old) were divided into two groups: tail suspended (TS) and controls (C). After 14 days of treatment testosterone levels were determined. On fixed sections histological stain (HE) was performed; DNA fragmentation was visualized using TUNEL technique. On the frozen samples immunoblotting for BAX, Bcl2, Caspase 39 and p53 was carried out. Testosterone levels were 0.18±0.09 and 3.74±2.2 ng/ml (mean±DS) in suspended and control animals, respectively. HE staining showed disturbed cell arrangement and a markedly decreased number of spermatozoa in the testes, TUNEL revealed an increased apoptotic index and immunoblotting provided evidence of apoptotic markers in TS animals. These data support results previously obtained by other groups showing a significant influence of short duration microgravity conditions on testicular function

Introduction

Life on earth have developed and evolved under the pressure of the earth gravitational fields. In the present millennium the conquest of Space will be pursued more and more and an increasing number of cosmonauts will be exposed to near zero gravity in long duration missions.

Knowledge about the effect of microgravity (μG) on the physiology of humans and other mammals is still in progress and much has still to be done. Researches on the effects of Spaceflights on reproductive system are almost absent in female and very few on male. Male dogs sent up on board of the satellite Cosmos 110 for 22 days showed an increase of 30-70% of atypical spermatozoa [1]. Relative immobilization during Space flight caused arrest of spermatogenesis in monkeys [2]. Rats flown in several missions showed a reduced testicular weight, a decrease in the number of spermatogonial cells [3] and in circulating testosterone [4, 5]. With regard to humans, a decrease in testosterone excretion was observed during Space flights [6, 7, 8]. In ground based studies, hind limb suspension (HLS), has been widely used in rats with partial constriction of the inguinal canal to prevent cryptorchism. In such experiments a marked decrease of circulating testosterone was observed after 7 days of HLS [9]. Long term HLS (6 weeks) experiment resulted in significantly reduced testicular weight and spermatogenesis [10]. At cellular level, studies on the effect of a reduction of gravity vector are very sparse. In previous investigations some of us observed damages to the cytoskeleton of testicular cells in a primary cell culture [11, 12]. Aim of the present study is to examine serum testosterone levels, testis morphology and apoptotic phenomena in tail suspended mice.

Materials and methods

Animals

Twelve male C57BL mice (6 months old, average weight 29.7 g) were divided into two groups: tail suspended (TS, facility HU, kindly provided by Prof. D. Conte Camerino, OSMA, University of Bari, Italy) and controls (C). The animals were housed at 24°C with a 12 hour light:dark cycle, drinking water and food were provided ad libitum. After 14 days blood was collected, the testes were excised and fixed in 4% paraformaldehyde or frozen at 20°C, one for each animal respectively.

DAPI staining

Fixed sections were stained with 4',6 diamidine 2-phenylindole hydrochloride (DAPI) (100 ng/ml in methanol)

for 1 min and observed concurrently at epifluorescence microscope.

TUNEL

DNA fragmentation was visualized in fixed sections by TUNEL (Terminal dUTP Nick End Labeling) method, in situ cell death fluorescein detection kit, Roche Diagnostic Co. Indianapolis, IN, USA).

Immunohistochemical techniques

Fixed testis sections (5 μm) were submitted to the indirect immunofluorescence technique. After exposure to Normal Goat Serum (diluted 1:50 in PBS; Sigma) in a moist chamber at 20°C, the sections were incubated overnight at 4°C with the the antiserum to 3β Hydroxysteroid dehydrogenase (raised in goat, against human type I, diluted 1:100 in PBS, Santa Cruz Biotechnology, Inc, CA, USA). After PBS washing (0.01 M, pH 7.4) a second layer of fluoresceineisothiocyanate conjugated globulins (FITC), donkey antigoat (diluted 1:100 in PBS, Santa Cruz Biotechnology, Inc, CA) for 30 min into a moist chamber, at 20°C. The slides were rinsed in PBS, mounted with gel-mount (Biomeda Corp., Foster City, CA). The specificity of the immunostainings was verified by omitting one of the steps of the immunohistochemical procedure, or by replacing the primary antisera with nonimmune rabbit serum or PBS. Immunoreactions were visualised using a conventional epifluorescence microscope.

Western blot analysis

Testes were washed twice in PBS and then homogenized in buffer containing 0,1% TritonX100 (SigmaAldrich, Milan, Italy). The protein extracts were quantified with a Bradfordbased colorimetric method (BioRad, Milan, Italy). Samples (20–30g for lane) were resolved on 12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and then transferred to a nitrocellulose membrane. Western immunoblot will be performed. The primary antibodies (anticaspase 3; anti caspase 9; anti p53; anti cytochome c Biotechnology Santa Cruz, CA, USA) will be detected with HRPconjugated antirabbit IgG diluted 1:10000. Blots will be developed using the One Step NBTBCIP staining solution (Perce Rockford, IL, USA).

Plasmatic Testosterone measurement

Serum total testosterone was measured in the blood serum using a solid phase enzymelinked immunosorbent assay (ELISA) (IBL International, Hamburg, Germany) as described by the manufacturer. The samples were run in duplicate with seven samples per group. Standards provided by the manufacturer were used: 0.2-16 ng/mL. Intraassay variation for this kit ranges from 3.34-4.16% (CV) while interassay variation is between 4.73-9.94% (CV). Sensitivity for total testosterone, was 0.083 ng/mL.

Results

HE staining showed disturbed cell arrangement and a markedly decreased number of spermatozoa in TS mice testes when compared to the sections from control animals. The extensive drop in mature spermatogenic cells in TS is confirmed by histological examination of the testes. Many tubules showed only spermatogonia and spermatocytes. In many cases seminiferous tubules lacking all spermatogenic cells were found next to tubules with some spermatogenesis. Almost no spermatozoa were observed. Normal spermatogenesis was observed in control animals (Fig. 1a, b). The TUNEL staining revealed that the apoptotic phenomena were significantly increased in the TS group compared with that in the control group (Fig. 2a, b). In contrast to the changes observed within the seminiferous tubules, the

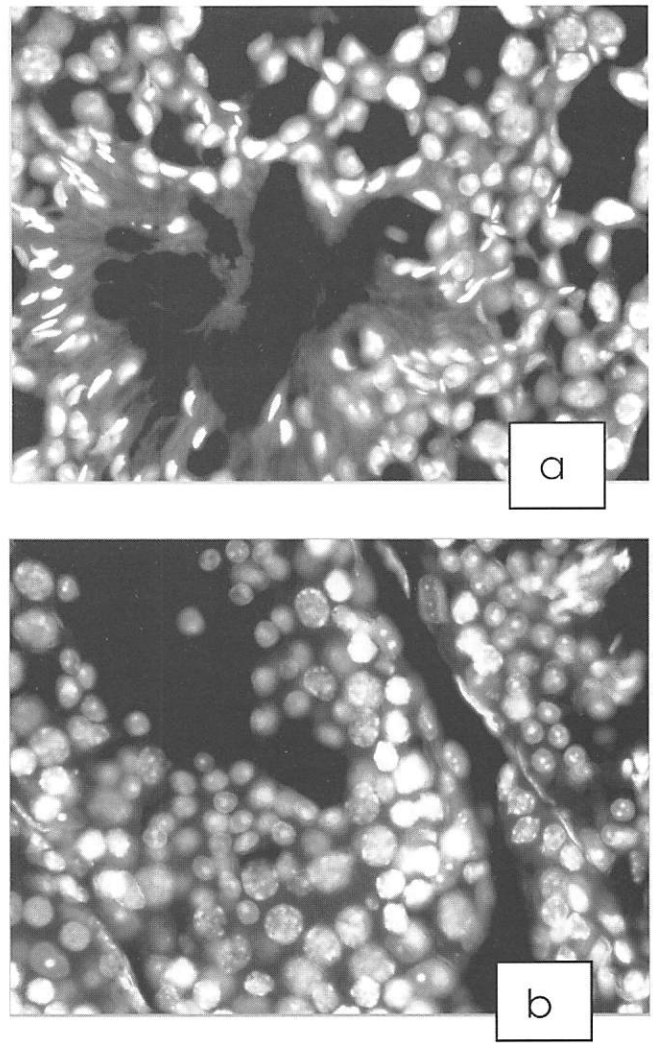

Fig. 1 – In the control testis (a) many spermatids are present, while the tubules are almost depleted in TS mice (b). In the TS mice testis the histology is highly disturbed.

interstitial region did not appeared to be affected in TS animals. However, in all TS animals immunostaining for 3β-HSD showed a number of positive Leydig cells lower in TS animals (Fig. 3a, b) and immunoblotting provided evidence of apoptotic markers in TS animals.

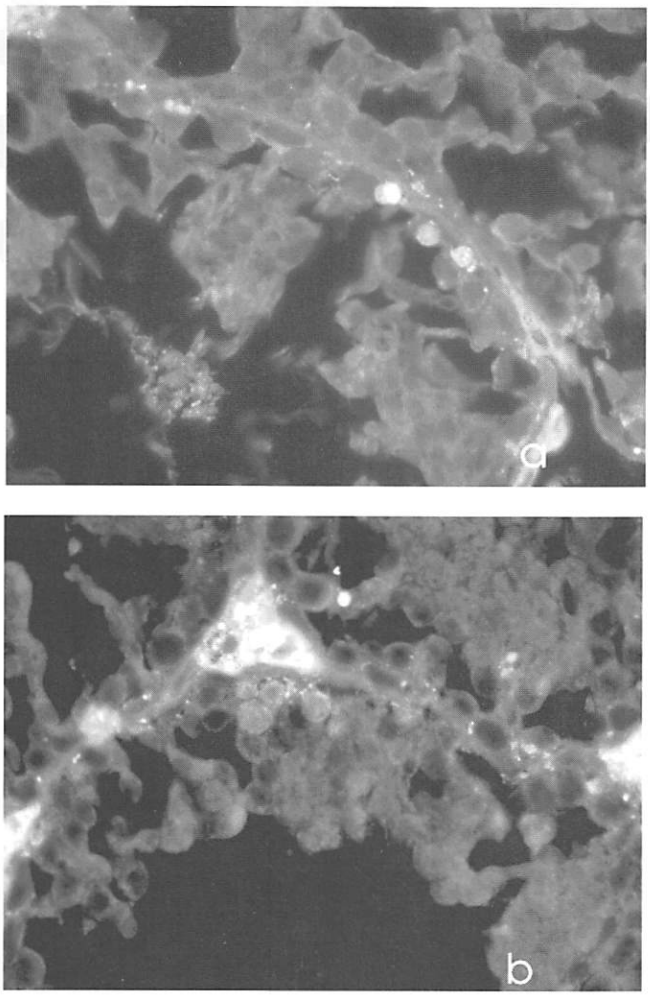

Fig. 2 – The TUNEL method revealed presence of DNA fragmentation in testis from TS mice (b), while in the control very few nuclei were stained (a).

Immunoblotting analysis demonstrated an increase in apoptotic markers (caspase 3, caspase 9, p53, cytochome c) in TS animals compared with the control animals (Fig. 4). The Testosterone concentration was significantly lower in TS group (0.18±0.09, mean±DS) than in the control group (3.74±2.2 ng/ml, mean±DS).

Discussion

As mankind enters the space age, whether humans can maintain a normal life cycle, reproduction, in Space becomes a critical problem. Reproduction experiments in real microgravity environments have been carried out with invertebrate and vertebrate specimens. A complete life cycle has been observed in Drosophila melanogaster [15]. Normal pregnancy and normal early embryogenesis occurred in killifish [16]. In mammals reports regarding the effect of Space flight on spermatogenic ability are still scarce and insufficient. In microgravity environment, we may observed a body fluid shift, so blood distributed in the upper half of the human body increases by about 2 L, causing a facial edema and cardiovascular responses, as well as disturbance in hormones and reproductive organs [5, 6].

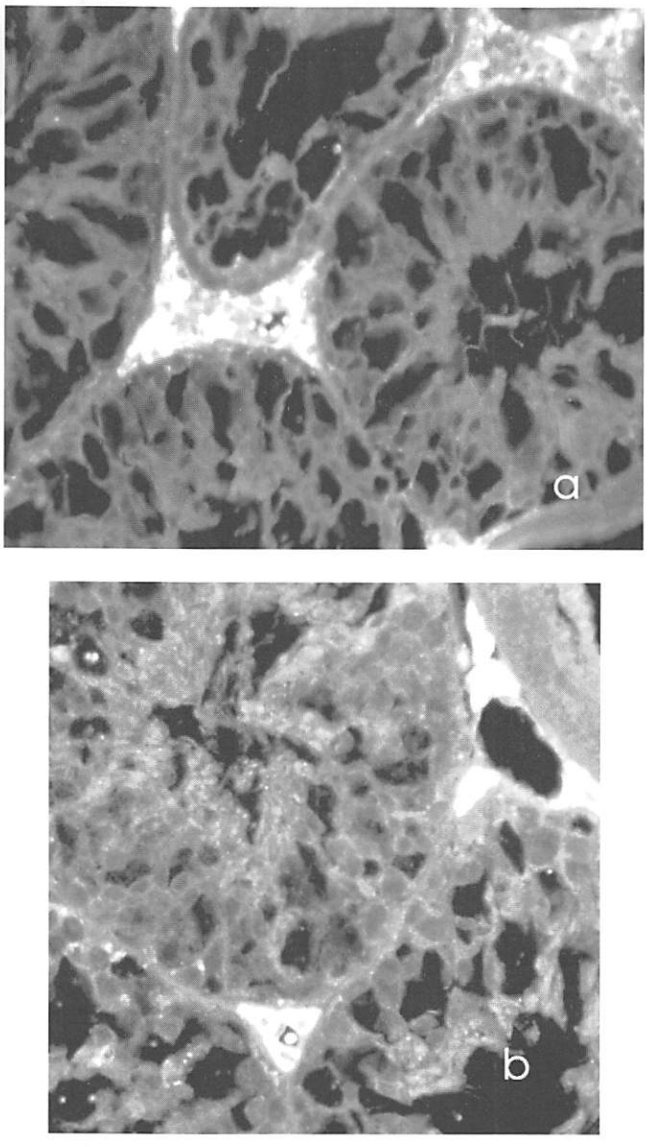

Fig. 3 – The immunofluorescence for 3βHSD is visible in numerous Leydig cells in control (a), few cells are present in testis from TS mice (b).

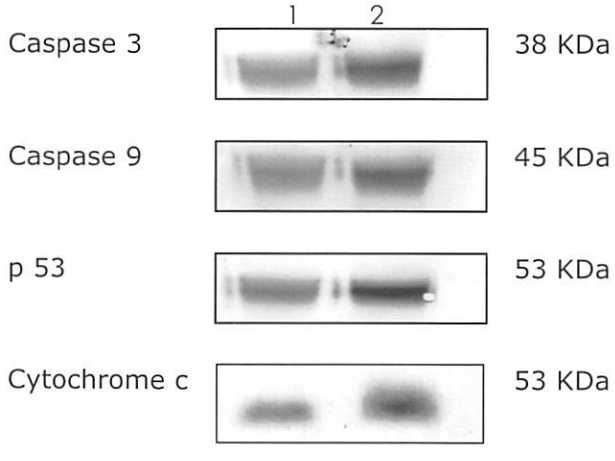

Fig. 4 – Western immunoblot analysis in testis lysates from control mice (lane 1) and HL mice (lane 2). The immunoreactions is stronger in treated rats.

The decrease in testosterone level after 14 days of tail suspension may be correlated to the reduced testicular blood flow in association with the cranial shift of body fluid. Tash et al [10]. demonstrated that after an early decrease in testosterone level, there is a recover by the end of 6 weeks of tail suspension. A compensatory mechanism can be hypothesized in long term experiments. An impairment in spermatogenic function in tail suspended mice was observed. Similar results were obtained after testosterone affecting drug exposure [17] or after trauma [18]. Therefore our results suggest to be affected by decrease in blood volume or changes in hormonal secretion. TUNEL staining and Wester immunoblotting results revealed an increase in apoptotic phenomena in TS mice, important to eliminate abnormal cells and maintain normal cells. Regulatory hormonal mechanism in spermatogenesis are reported to affect apoptosis [19]. Our experiment indicate that after 14 days of tail suspension a testicular atrophy is evident and apoptosis of germ cells increases. In conclusion a microgravity environment appears may affect spermatogenesis. Because TS model is widely accepted as a model for μG, it will be important to determine the mechanism that underlie the reduction of spermatogenesis. If our findings holds true in μG, it implies that male astronauts may become infertile after long term weightlessness exposure.

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