

# Simulated microgravity activates apoptosis and NF- $\kappa$ B in mice testis

Chidananda S. Sharma · Shubhashish Sarkar · Adaikkappan Periyakaruppan · Prabhakaran Ravichandran · Bindu Sadanandan · Vani Ramesh · Renard Thomas · Joseph C. Hall · Bobby L. Wilson · Govindarajan T. Ramesh

Received: 17 January 2008 / Accepted: 13 March 2008 / Published online: 4 April 2008  
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**Abstract** Microgravity is known to have significant effect on all aspects of reproductive function in animal models. Recent studies have also shown that microgravity induces changes at the cellular level, including apoptosis. Our effort here was to study the effect of simulated microgravity on caspase-8 and the caspase-3 activities, the effectors of the apoptotic pathway and on the transcription factor NF- $\kappa$ B a signaling molecule in mouse testis. Morey-Holton hind limb suspension model was used to simulate microgravity. Caspase-8 and 3 fluorometric assays were carried out and HLS mice testis exhibited a 51% increase in caspase-8 and caspase-3 compared to the controls. A sandwich ELISA-based immunoassay was carried out for detection of NF- $\kappa$ B which again significantly increased in the test mice. Testosterone levels were measured using an ELISA kit and in HLS mice the decrease was significant. There was also a significant decrease in testis weight in the test mice. Simulated microgravity activates caspase 8, 3 and NF- $\kappa$ B necessary to stimulate the apoptotic pathway in mice testis. This may account for the drop in testis weight and testosterone level further affecting testicular physiology and function.

**Keywords** Microgravity · Apoptosis · Caspase · NF- $\kappa$ B · Testosterone

## Introduction

Reproduction in animals including humans is a well-adopted phenomenon within the earth's gravitational field (1-g). Any deviation in the gravitational force may have a profound effect on reproductive physiology and fertility of the subject [1]. Astronauts experience both microgravity (weightlessness) effect during the time of orbit and hyper-gravity during landing. Hyper-gravity experienced by astronauts is normally for a very short duration and probably has limited effect on biological functions. The effect of microgravity on reproductive functions carried out until today is hampered because of the short duration, high costs, and limited opportunities of space flight travel. Further the responses to decreased gravity during orbit versus increased gravity during landing are difficult to separate because the subjects are immediately not recovered after flight. In the era of the international space station with eventual goal of space colonization the emphasis on space biology research from acute responses to chronic effects of altered gravity is required [2]. If spaceships need to be successfully colonized, then a more detailed study and comprehensive understanding of the effects of altered gravity on mammalian reproduction is required.

Extensive reproductive studies are therefore needed using animal and cell culture models to establish safety guidelines for space travel. Scientists are facing challenges in ground-based models in gravitational biology conducting space flight experiments with animal models, as it is very difficult to exactly simulate the space travel. During space travel reproductive changes that occur in humans may be due to gravity or other conditions aboard the spacecraft such as increased radiation, noise, isolation, disrupted circadian rhythms, and stress. The most popular ground-based rodent model for studying hypo-gravity (microgravity) is

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C. S. Sharma · P. Ravichandran · V. Ramesh · J. C. Hall · G. T. Ramesh (✉)  
Department of Biology, Molecular Toxicology Laboratory,  
Center for Biotechnology & Biomedical Sciences,  
Norfolk State University, Norfolk, VA 23504, USA  
e-mail: gtramesh@nsu.edu

S. Sarkar · A. Periyakaruppan · B. Sadanandan · R. Thomas · B. L. Wilson · G. T. Ramesh  
NASA University Research Center, Texas Southern University,  
Houston, TX 77004, USA

Morey-Holton hind limb suspension (HLS model). In this HLS model animals are suspended by the tail base to produce 30° head down position that complements the human 6° head down tilt utilized in bed rest studies [3, 4]. Suspension of rats in a special harness (caudal elevation) to induce thoracic pooling of blood fluids and prevention of support function of the hind limbs is used to mimic, on earth, the effects of microgravity encountered during space flight, which induces cryptorchidism in male rats. Using HLS model, experiments can be scheduled with low cost; manipulations can be done at different time intervals in a single experiment on earth. Further, experiments can be repeated and extended on routine basis and tissues can be collected from anesthetized animals at any time of experimentation with cost effective, ease, and without riding the space flight [4].

By studying the effect of microgravity on reproductive functions in rodent models, the scientists can understand the aspects of human reproductive physiology and fertility. Such studies also help to develop counter measures to maintain normal reproductive and physiological functions during space flight. In males, testis is an important organ of the reproductive system. Testis carries out dual functions, spermatogenesis and the steroidogenesis. In steroidogenesis testosterone the principal androgen produced in testis maintains male reproductive organ function and stimulates the sperm production in adult males. Very few space travel studies carried out reported the interesting finding that there was a transient dramatic reduction in steroidogenesis and therefore reduced testosterone levels in humans and rats [5–8]. Few studies carried out on animals subjected to simulated microgravity also reported the reduction in testis weight with decreased levels of testosterone hormone levels both in testis and plasma [6–8]. However, one study reported that simulated microgravity has no effect on testosterone levels in testis and plasma [9]. These conflicting reports prompted us to study the effect of simulated microgravity on the testis and testosterone hormone levels. Further the effect of simulated microgravity on caspase-8 and 3 enzyme activities, the effectors of the apoptotic pathway were investigated. Simulated microgravity and its effect on the transcription factor NF- $\kappa$ B which acts as a signaling molecule at cross roads of many pathways such as inflammation, proliferation, and apoptosis [10] were also addressed.

## Materials and methods

### Animal and chemicals

Six to eight week BALB/c male mice (15–20 g body weight) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Testosterone ELISA kit and NF- $\kappa$ B colorimetric enzyme immuno-assay kit were obtained from Neogen

Corporation (Lexington, KY). Caspase-3 and caspase-8 enzyme apoptosis detection kits (sc-4263 AK sc-4267 AK, respectively) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Other chemicals sodium mono hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride used for phosphate buffer saline were of analytical grade and obtained from Fisher Scientific (Suwanee, GA).

### Animal maintenance and Hind limb suspension of mice for simulated microgravity

The standard animal protocols for maintenance of mice and hind limb suspension protocols used were approved by the Institute Animal Welfare Committee of Texas Southern University (Houston, TX). Male mice obtained from Harlan (Indianapolis, IN) were maintained on a 12-h light and dark cycle. The animals were housed in individual cages in a temperature controlled room at  $21 \pm 2^\circ\text{C}$  and provided with standard food pellet and water available ad libitum. Three days after arrival, the animals were randomly assigned as control mice group ( $n = 6$ ) and tail suspended mice as experimental group ( $n = 6$ ). Unloading tail suspension of the mice was carried out essentially as previously described [4, 11–13]. In brief the tail of the animals were cleaned and dried. A thin layer of adhesive tape was applied approximately to the middle of the tail along the medial to lateral sides and harness made of standard porous tape was pressed firmly to the glue. A paper clip was used to attach the animal's tail harness to a swivel apparatus on the wire spanning the top of a cage. The height of the animal's hind limbs was adjusted to prevent any contact with the cage bottom, which gives a tilt of 30° head downwards. The fore limbs of the animal maintained contact with the cage bottom allowing the rat full access to the entire cage. The control animals were kept individually in identical cages and allowed to move freely all over the cage. Weight of each animal was measured on a daily basis until the day of completion of the experiment.

### Animal euthanization, testis organs collection

Following HLS of mice, on the 12th day both control and HLS animals were euthanized with isofluorine. The testis was collected from all the animals and weights were measured. The organs were stored at  $-80^\circ\text{C}$  until used for further experiments.

### Testosterone measurements in testis samples

The testes were suspended in five volumes of buffer 50 mM Tris-HCl, pH 7.4 containing 10 mM EDTA, 10 mM EGTA, 1 mM DTT and homogenized. The samples were centrifuged at 800g for 5 min to remove cell debris. The supernatant was further centrifuged at 10,000g for 10 min. The cytosolic

fraction was used for testosterone and caspase activity measurements. Testosterone in testis samples were measured according to the method described earlier and based on the instructions given in the kit [14]. Extraction of testosterone in testis homogenate samples was carried out in diethyl ether. Testis homogenate samples (25  $\mu$ l) in a 10-ml glass tube were diluted with extraction buffer and 1 ml of diethyl ether was added and extracted by vortexing. The tubes were frozen in liquid nitrogen and the organic layer was transferred to clean glass tubes and by passing a stream of nitrogen diethyl ether in the samples was evaporated. The residue was dissolved in 100  $\mu$ l of PBS and suitably diluted and used for testosterone assay. The assay operates on the basis of competition between the enzyme conjugate and the testosterone in the samples for a limited number of binding sites on the antibody-coated plate. The sample or standard testosterone solution and diluted enzyme conjugate was added to the antibody-coated microplate and incubated at room temperature for 1 h on a shaker. The plate was extensively washed with washing buffer. The bound enzyme conjugate was detected by the addition of substrate, which generates optimal color after 30 min. The OD was read at 650 nm. The extent of color is inversely proportional to the amount of testosterone in the sample. The results were normalized per mg of protein.

#### Caspase-8 activity assay

Caspase-8 fluorometric assay was carried out on the principle of hydrolysis of IETD-AFC a synthetic substrate as described earlier [15, 16]. IETD-AFC corresponds to the upstream amino acid sequence of the caspase-8 cleavage site in caspase-8 and the fluorophor AFC (7-amino-4-trifluoromethyl coumarin). Proteolysis between D amino acid and AFC by caspase-8 releases AFC. AFC emits a yellow-green fluorescence at 480–520 nm (peak at 505 nm) excited at 400 nm. Apoptotic cells activate caspase-8 hence emit high levels of fluorescence compared to control non-apoptotic cells. The assay was carried out in 96-well plate. Equal volumes of testis homogenate from both control and experimental animals were diluted with reaction buffer and DTT was added to a final concentration of 10 mM. To the reactant mixture 5  $\mu$ l of IETD-AFC substrate was added and incubated for 1 h at 37°C. Free AFC levels formed were measured in a plate reader with a 400 nm excitation and a 505 nm emission. The results of experimental samples were compared with control and expressed as fluorescence units/mg of protein.

#### Caspase-3 activity assay

The principle of Caspase-3 fluorometric assay is the same as caspase-8 assay except that a different DEVD-AFC synthetic substrate was used [15, 16]. DEVD-AFC

corresponds to the upstream amino acid sequence of the caspase-3 cleavage site in PARP and the fluorophor AFC (7-amino-4-trifluoromethyl coumarin). Caspase-3 hydrolyzes the peptide bond between D amino acid and AFC releasing AFC. AFC emits a yellow-green fluorescence at 480–520 nm (peak at 505 nm) excited at 400 nm. Apoptotic cells activate Caspase-3 hence emit high levels of fluorescence compared to control non-apoptotic cells.

The procedure was carried out in 96-well plate with 10  $\mu$ l of testis homogenate from both control and experimental animals diluted in reaction buffer, with DTT to final concentration 10 mM. To the reactant mixture 5  $\mu$ l of DEVD-AFC substrate was added and incubated for 1 h at 37°C. Free AFC levels formed were measured in a plate reader with a 400 nm excitation and a 505 nm emission. The results of experimental samples were compared with control and expressed as fluorescence units/mg of protein.

#### NF- $\kappa$ B binding assay

NF- $\kappa$ B colorimetric enzyme immunoassay was carried out essentially as described previously. The principle of the assay was based on sandwich type ELISA which employs an oligonucleotide containing a DNA binding NF- $\kappa$ B consensus sequence. Samples or NF- $\kappa$ B standards were added to the wells and incubated at room temperature for 1 h. NF- $\kappa$ B present in the sample binds specifically to the oligonucleotide coated on the plate. Wells in the plate were extensively washed with washing buffer. Specific antibody (100  $\mu$ l) was added to all the wells and incubated further for 1 h. The DNA-bound NF- $\kappa$ B selectively binds to the primary antibody (p50 and p105 specific). The plate was washed with washing buffer and the antibody-bound NF- $\kappa$ B bound to secondary antibody–alkaline phosphatase conjugate on incubating for 1 h at room temperature. To the washed plate TMB substrate was added and incubated for 30 min and the OD was measured in a colorimetric plate reader at 450 nm. The results were expressed as pg of NF- $\kappa$ B/ $\mu$ g of protein.

#### Statistical analysis

Data were expressed as mean  $\pm$  SD and statistical significance was assessed by student's *t*-test. Difference between control and simulated microgravity samples was considered significant if the level was  $P < 0.05$ .

## Results

#### Effect of simulated microgravity on body weight and testis weight

Body weights of all the animals were taken on a daily basis. The body weight on the day of sacrifice was

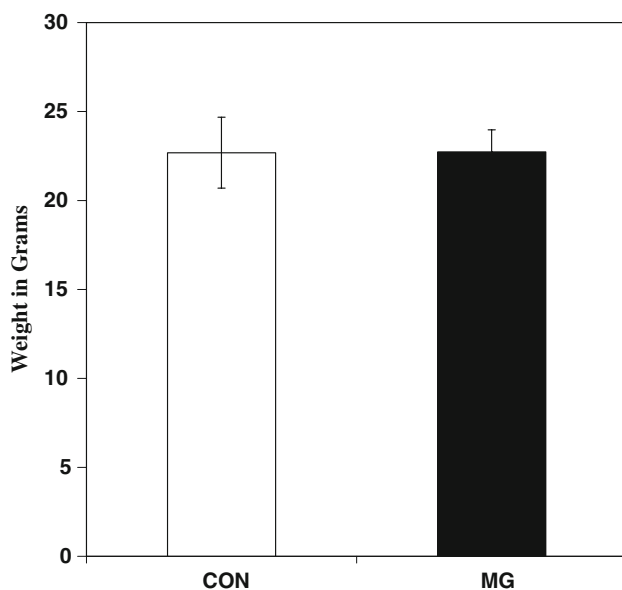
presented in Fig. 1. Average body weight of simulated microgravity subjected mice after 12 days of hind limb suspension was found to be not significantly different from control mice (Fig. 1). However, the testis weight of simulated microgravity subjected mice decreased by 41%, which was found to be statistically significantly different from the control mice (Fig. 2).

#### Effect of simulated microgravity on testosterone content in testis

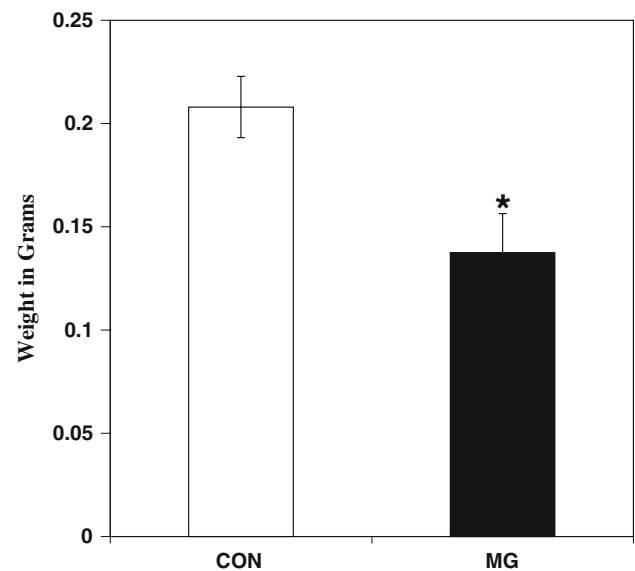
Testosterone the principal steroid present in testis significantly decreased in simulated microgravity subjected mice. There was 46% decrease in the testosterone levels in HLS mice testis compared to control animals (Fig. 3).

#### Effect of simulated microgravity on caspase-8 and caspase-3 activities in testis

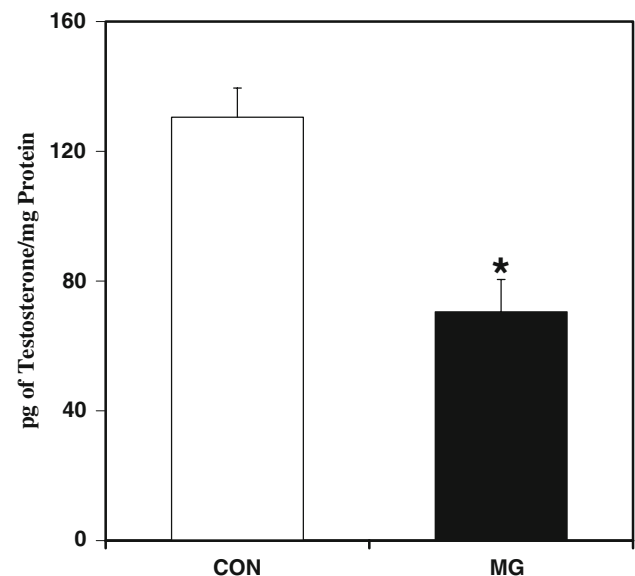
Procaspase-8 activation to caspase-8 the initiator of apoptosis was measured by fluorometric assay clearly suggested that microgravity subjected mice show 140% increased activity compared to control mice (Fig. 4). The results obtained were found to be statistically significant. The activation of caspase-3 a downstream effector caspase was also found to be activated in HLS mice testis compared to controls. The activation was found to be 51% increase in microgravity subjected mice compared to controls (Fig. 5).



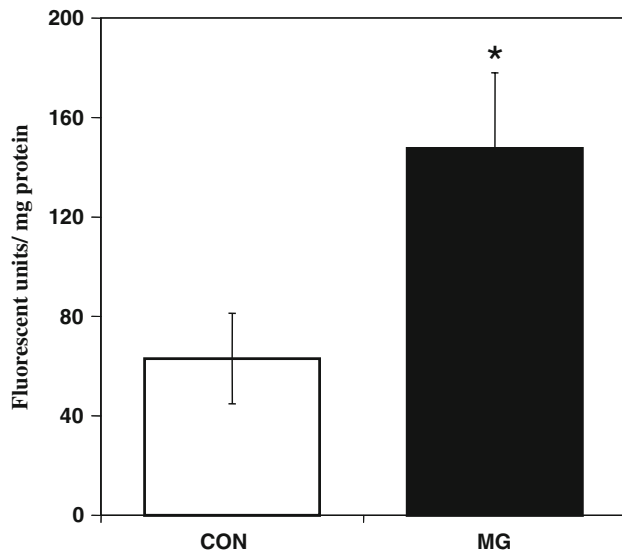
**Fig. 1** Effect of simulated microgravity on the body weight of mice. Average body weight expressed as grams of simulated microgravity (MG) subjected mice ( $n = 6$ ) and control (Con) mice ( $n = 6$ ) after 12 days hind limb suspension. The results of HLS mice were found to be not significantly different from control mice. The figure is a representative from three experiments performed independently



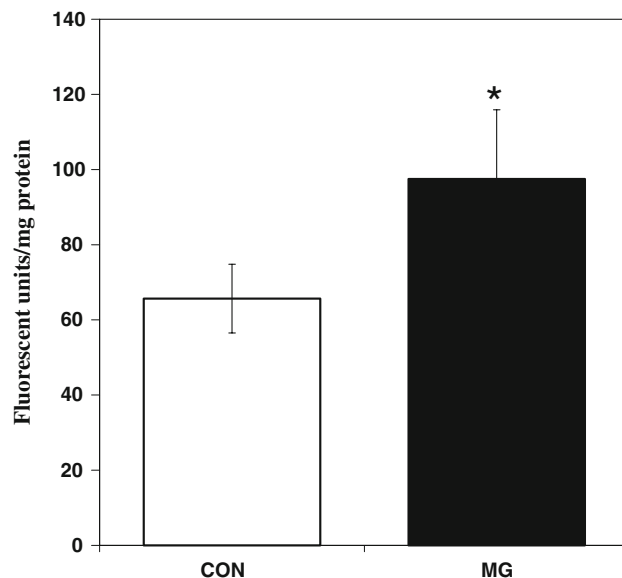
**Fig. 2** Effect of simulated microgravity on the testis weight of mice. Average testis weight expressed as grams/pair of testis/animal of simulated microgravity (MG) subjected mice ( $n = 6$ ) and control (Con) mice ( $n = 6$ ) after 12 day hind limb suspension. The results of HLS mice were found to be significantly different from control mice ( $*P < 0.01$ ). The figure is a representative from three experiments performed independently



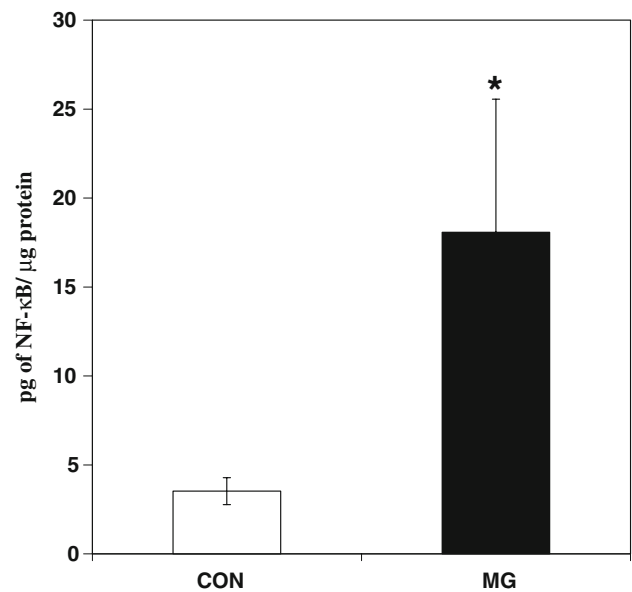
**Fig. 3** Effect of microgravity on testosterone concentration in mice testis. The sample or standard testosterone solution and diluted enzyme conjugate was added to the antibody-coated microplate and incubated at room temperature for 1 h. The plate was washed and the bound enzyme conjugate was detected by the addition of substrate. After 30 min the OD was read at 650 nm. The results were expressed as pg of testosterone/mg protein. The results of HLS mice were found to be significantly different from control mice ( $*P < 0.01$ ). The figure is a representative from three experiments performed independently



**Fig. 4** Effect of simulated microgravity on caspase-8 activity in mice testis. Caspase-8 fluorometric assay was carried out on the principle of hydrolysis of IETD-AFC a synthetic substrate. AFC released by caspase-8 emits a fluorescence. The assay was carried out in 96-well plate. Testis homogenate and IETD-AFC substrate was added and incubated for 1 h at 37°C. AFC formed were measured in a plate reader with a 400 nm excitation and a 505 nm emission. The results were expressed as fluorescence units/mg of protein. The results of HLS mice were found to be significantly different from control mice (\* $P < 0.001$ ). The figure is a representative from three experiments performed independently



**Fig. 5** Effect of simulated microgravity on caspase-3 activity in mice testis. The principle of Caspase-3 fluorometric assay is exactly same as caspase-8 assay except that a different DEVD-AFC a synthetic substrate was used. The assay was carried out in 96-well plate. Testis homogenate and DEVD-AFC substrate was added and incubated for 1 h at 37°C. AFC formed was measured in a plate reader with a 400 nm excitation and a 505 nm emission. The results of HLS mice were found to be significantly different from control mice (\* $P < 0.05$ ). The figure is a representative from three experiments performed independently



**Fig. 6** Effect of simulated microgravity on NF-κB activation in mice testis. NF-κB sandwich type ELISA was used. Samples or NF-κB standards were incubated in 96-well plate. NF-κB present in the sample binds specifically to the oligonucleotide a DNA binding NF-κB consensus sequence-coated wells. NF-κB antibody was added to the wells and incubated further. The antibody-bound NF-κB was detected by secondary antibody–alkaline phosphatase conjugate and TMB substrate in an ELISA reader at 450 nm. The results were expressed as pg of NF-κB/μg of protein. The results of HLS mice were found to be significantly different from control mice (\* $P < 0.01$ ). The figure is a representative from three experiments performed independently

#### Effect of simulated microgravity NF-κB in testis

It is a well-known fact that transcription factor NF-κB and IKK proteins regulate many physiological processes. To understand the effect of microgravity on NF-κB and its role in apoptotic pathway the binding activity NF-κB was measured in testis of HLS mice and control mice. The results of such a study suggested that there was a significant, 133% increase in the binding activity of the NF-κB in HLS mice testis compared to controls (Fig. 6).

#### Discussion

HLS mouse model was used to study the effect of simulated microgravity on the physiology and function of testis. A variety of biological processes are perturbed in mice when exposed to microgravity (space flight) for more than 7 days, including testicular functional changes. It is a well-known phenomenon that microgravity induces oxidative stress and such stress leads to multiple signals and varied responses in different organs of the subjects [17]. The results of the present study show that simulated microgravity affects the steroidogenesis pathway in leydig cells

of mice by an unknown mechanism yet to be established. The steroid may be involved in the development and maintenance of physiological functions of testis, hence testis weight decreased. Other groups also made similar observations in different animal models of simulated microgravity. In studies carried out in rat suspended model a significant decrease in testosterone levels and testis weight was also observed [18, 19]. Decrease in the testosterone levels may have an overall influence on the physiological function of testis and hence decrease in weight was observed. Treatment of adult rats with luteinizing hormone (LH) resulted in hyperplasia, hypertrophy, and increased testosterone secretory capacity of leydig cells [20]. Therefore the simulated microgravity may have direct effect on the pituitary LH synthesis or it may have direct effect on steroidogenic pathway. Studies carried out on in vivo administration of triiodo thionine (T3) during first week of pregnancy in rats caused increase in testis size by more than 60%. In vitro T3 treatment on culture of neonatal testis fragment significantly increased in the size of seminiferous cords and number of gonocytes [21]. In transient hypothyroidism also rat's testicular size and sperm production increased [22]. Thus hind limb suspension may have an effect on thyroid function, which may have regulatory function on the testicular growth that also needs to be addressed. Following 28 day tail suspension, in addition to the testis weight loss and decreased testosterone content, the sperm count and sperm motility found to be significantly decreased [19]. The seminiferous tubules show atrophy with the reduction in the layers of seminiferous epithelium and decreased in sperm numbers in lumen of seminiferous tubules probably due to apoptosis [19]. Sertoli cells carry Fas receptor and Fas ligand, while the germ cells carry only Fas receptor. The Fas ligand released by Sertoli cells may induce apoptosis of Sertoli cells in an autocrine mode or induce apoptosis of germ cells in a paracrine mode [23, 24]. Signaling of apoptosis may proceed in two ways, one through recruitment of FADD (Fas associated death domain protein) and activation of procaspase-8. Caspase-8 in turn activates other downstream caspase-3, caspase-6, and caspase-7. Alternatively through cytochrome-c released from mitochondria assembles itself with apoptosome complex that leads to the activation of procaspase-9 and subsequent activation of downstream caspases leading to apoptosis of the cells [25]. Our data clearly suggest that apoptosis was induced in hind limb suspended mice testis as the activity of caspase-8 was significantly increased. Caspase-8 is the key initiator (activator) first to be activated in apoptotic death receptor pathway, when the cells commit to die. Ligands upon binding to the receptor such as CD-95 (Apo/Fas) aggregate and form membrane-bound complex. This complex via adaptor molecules of Fas associated death domain (FADD)

recruit several molecules of procaspase-8 which auto activates to caspase-8 [26].

Our study further shows that the effector caspases are also activated in hind limb suspended mice testis as the downstream caspase-3 was also significantly increased. In vitro studies using human Sertoli cells have shown that activation of caspase enzyme occurring in response to testosterone withdrawal DNA fragmentation [27] agrees well with our in vivo model where decreased testosterone content in testis activates caspases the principal players of apoptosis. Even though in our study, we have not addressed individual cell types, as the testis size decreased considerably, it may be hypothesized based on previous studies that major cell type Sertoli cells undergo apoptosis as there was decreased testosterone levels in testis. By autocrine action of apoptotic pathway through Fas ligand caspase-8 and caspase-3 enzymes were activated leading to death of Sertoli cells. A recent study demonstrated the Sertoli cell cytoplasmic degradation and inappropriate cell associations in Chimpanzee seminiferous tubules following 21 days of in vivo androgen deprivation [28].

In vitro studies using human Sertoli cells clearly suggested that these cells are primarily involved in the regulation of apoptosis in the adult human testis [27]. Apoptosis in germ cells induced in vitro by testosterone withdrawal occurred at a later stage as a secondary phenomenon following the action in Sertoli cells as germ cells are believed to lack a functional FSH receptor [29]. Our in vivo study reflects those studies carried out in vitro, with withdrawal of testosterone and represents an acute response or may be a more generalized caspase activation involving different stages of germ cells. In fact caspase-3 activation has been shown to occur in germ cells after a week of in vivo reduction of intracellular testosterone and inter-nucleosomal DNA cleavage in germ cells continued increasing through the subsequent 3 weeks [30]. Germ cell apoptosis occurring as an acute response to hormone withdrawal is not mediated by caspases. The question that arises here is the biological significance of the upregulation of Fas receptor in rat [31] and human [32] germ cells occurring after in vitro induction of massive cell death. This is also found in spermatids from patients with post-meiotic maturation arrest [23].

Previous studies demonstrated that testicular germ cell apoptosis was induced by androgen withdrawal [33–35], or gonadotropin withdrawal or by heat stress [36]. In a study with 1, 3-dinitrobenzene (1,3-DNB), a testicular toxicant, was given as a single oral dose (25 mg/kg body weight). Histopathological examinations revealed the damage of Sertoli cells, a marked increase in the number of apoptotic pachytene spermatocytes in seminiferous tubules. Earlier studies carried out on ultrastructural examinations of the testis of rat administered with a single oral dose of 1,3-dinitro benzene, observed testicular lesions limited to stages VIII–XI of the spermatogenic cycle following 12 h

treatment [37]. It was suggested that the Sertoli cells were implicated as the primary target for the toxic action of 1,3-DNB, followed by the germ cell damage as a secondary event [37]. In testis treated with 1, 3 DNB the Bax pro-apoptotic gene was also up regulated in pachytene spermatocytes [38]. Based on the above studies, we could also say that in our simulated microgravity model, morphological damage occurred by the activation of apoptosis through Fas ligand binding to its receptor at the Sertoli cell level and by the paracrine action of Fas ligand activating the germ cell apoptosis. However, detailed analysis and microdissection of different cell types of the HLS mice testis is required to understand the effect of simulated microgravity on apoptosis pathway.

Our studies also show that NF- $\kappa$ B is activated in the testis of mice subjected to simulated microgravity. The anti-apoptotic functions, that is tumor promoting abilities of NF- $\kappa$ B and Rel-A have been well established [39]. However numerous reports also suggest that NF- $\kappa$ B performs a pro-apoptotic role. NF- $\kappa$ B/Rel-A complex can directly stimulate the expression of apoptosis-inducible genes such as Fas, Fas-ligand, and death receptors 4 and 5 [39]. Rel-A represses the expression of certain anti-apoptotic genes such as Bcl-xL in response to NF- $\kappa$ B atypical inducers such as UV-C and daunorubicin [40–42]. TNF- $\alpha$  which induces NF- $\kappa$ B pathway, can also activate Jun n-terminal kinase (JNK), which has pro-apoptosis effects [43, 44]. There may be a cross talk between NF- $\kappa$ B and p53 a tumor suppressor protein which regulates apoptotic pathway. The amount of p53 protein in cells is regulated by HDM2 (known as MDM2 in mice). HDM2 which functions as E3 ubiquitin ligase can induce p53 proteolysis [45]. Further stimuli which induces NF- $\kappa$ B or I $\kappa$ B family member BCL-3 also can induce expression of HDM2 and reduce p53 protein levels [10]. Thus in testis reduced gravity which activates NF- $\kappa$ B probably activates MDM2 or Fas or Fas ligand or death receptors and hence apoptosis is activated. The role of NF- $\kappa$ B in the activation of above pro-apoptotic genes or repression of anti-apoptotic genes in different cell types of mouse testis under microgravity situation needs to be investigated. In vitro phenomenon the effect of testosterone withdrawal on human sertoli cell function and interdependent of different cell types on testicular functions can be addressed in animal hind limb suspension models especially in rodents.

**Acknowledgment** This work was supported by NASA NCC 9-165: NIH 1P20MD001822-1 (GR).

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