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In vitro effects of simulated microgravity on Sertoli cell function

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Abstract

With the advent of space flights questions concerning the effects of microgravity (0×G) on human reproductive physiology have received great attention. The aim of this study was to evaluate the influence of 0×G on Sertoli cells. A Sertoli cell line from mouse testis (42GPA9) was analyzed for cytoskeletal and Sex Hormone Binding Globilin (SHBG) changes by immunohistochemistry, for antioxidant content by RT-PCR and for culture medium lactate concentrations by protein chemistry. Cells were cultured for 6, 24 and 48 h on a three-dimensional Random Positioning Machine (3D-RPM); static controls (1×G) were positioned on the supporting frame. At the end of each experiment, cultured cells were either fixed in paraformaldehyde or lysed and RNA-extracted or used for culture medium lactate measurements as needed. At 0×G, Sertoli cytoskeleton became disorganized, microtubules fragmented and SHBG undetectable already after 24 h, with alterations worsening by 48 h. It was evident that various antioxidant systems appreciably increased during the first 24 h but significantly decreased at 48 h. No changes occurred in the 1×G samples. Initially, 0×G seemed to disturb antioxidant protection strategies allowing the testes to support sperm production, thus generating an aging-like state of oxidative stress. Lactate production at 0×G slightly decreased after 24 h. Further experiments are needed in space to investigate upon steroidogenesis and germ cell differentiation within the testis, to rule out male infertility as a possible consequence, which could be a problem, as life expectancy increases.

Keywords: Sertoli cells; Microgravity; Aging; Antioxidant agents; Cytoskeleton

1. Introduction

Life on earth has developed and evolved under the pressure of earth's gravitational field. Studies on the effect of gravity vector changes on the physiology of humans and other animals have been neglected in the past because of the relative consistency of the gravitational field in our planet and the impossibility of obtaining lower or higher gravity conditions elsewhere. With the advent of space flights/stations and future long term missions aimed at reaching and colonizing distant planets in our Solar System including Mars, the dream to explore outer spaces in the Galaxy has become plausible and therefore questions concerning the effects of different gravitational forces on human physiology have become impelling. Astronauts are exposed to hypergravity during launch and landing and near zero-gravity during the missions. In fact, hypergravity is experienced for a very short period of time, and therefore, has a limited influence on physiological functions, whereas, long-term microgravity may have more relevant effects (Lazerges, 1990; Sumanasekera et al., 2006; Pecaut et al., 2004).

In the era of the International Space Station with the eventual goal of space colonization, biology research upon acute responses through chronic effects of altered gravity has to be emphasized (Moody and Gorwill, 2000). If space

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stations and planets need to be successfully colonized, then a more detailed study is required in order to understand the effects of altered gravity on mammalian reproduction is required. Therefore, extensive reproductive studies are needed, by first using animal and cell culture models, in order to establish safety guidelines for space travel.

There are few studies concerning the effects of space flights on the reproductive system, especially in the female. Male dogs onboard the Cosmos 110 satellite for 22 days showed a 30–70% increase in atypical spermatozoa (Federova, 1967). Relative immobilization during space flight caused the arrest of spermatogenesis in monkeys (Zemjanis et al., 1970; Merrill et al., 1992). In the rats that participated in several missions, both testicular weight and the number of spermatogonial cells decreased (Philpott et al., 1985) and lower circulating testosterone levels were found (Grindeland et al., 1990). In humans, a decrease in testosterone secretion was observed during space flights (Stein and Schluter, 2000; Strollo et al., 2004; Strollo, 1993).

Scientists are faced with challenges when using ground-based microgravity simulation methods with animal models, since it is impossible to take into account all the variables involved in real flights. In fact, reproductive changes occurring in humans during space travels may be due to gravity, as well as, to other conditions aboard the spacecraft such as: increased radiation, noise, isolation, disrupted circadian rhythms, and stress. A modeled approach is needed to plan the extremely infrequent real flight experiments efficiently.

In ground based studies, hind limb suspension (HLS) has been widely used in rats with partial constriction of the inguinal canal to prevent cryptorchidism. In such experiments, a marked decrease of circulating testosterone was observed after 7 days of HLS (Amann et al., 1992). Long term HLS (6 weeks) experimentation resulted in significantly reduced testicular weight and spermatogenesis (Tash et al., 2002).

At the cellular level, there are very few studies regarding the effect of a reduction of gravity vector. Microgravity has proven to be one of the stress environmental factors that causes severe damage to the cytoskeleton of cells kept in culture (Uva et al., 2002; Sciola et al., 1999; Infanger et al., 2006). It has been reported that gravity vector changes damage the skeleton of lymphocytes both during space flight and in simulated weightlessness (Cogoli-Greuter et al., 1994; Gmunder et al., 1990). In our previous investigations, we observed damages to the cytoskeleton of testicular cells in a primary cell culture (Strollo et al., 1998; Uva et al., 2007). However, in these previous studies crude preparations of testicular cells were used.

Spermatogonial cell differentiation into mature spermatozoa requires Sertoli cells. These cells play a crucial role in the development of germ cells and in the regulation of spermatogenesis. In addition to its nursing function, Sertoli cells produce a large number of proteins essential for germ cell survival and development such as transferring and lactate dehydrogenase (Griswold, 1993). The Androgen-Binding Protein (ABP) is among them. It is a steroid carrier to germ cells, and has the same amino acid sequence as the hepatic Sex Hormone Binding Globulin (SHBG) (Munell et al., 2002). Its secretion is considered a good marker for Sertoli cell function (Munell et al., 2002).

There is also evidence that an antioxidant system is effective within the testis to protect somatic and germ cells from oxidative damage through multiple enzymatic systems and antioxidant molecules. They include superoxide dismutase (SOD, transforming O_2 free radicals into H_2O_2), glutathione transferase (GST, conjugating damaging molecules with glutathione), catalase oxido-reductase (decomposing H_2O_2) and metallothioneins (MTs, playing a major role in antioxidant processes) (Aitken and Roman, 2008). In addition, PoliADP-ribose polymerase (PARP, activating DNA repair programs) and p53 protein (the guardian of the genome) may also be involved.

The aim of this study was to evaluate the influence of simulated microgravity on a Sertoli cell line from mouse testis using immunohistochemistry, molecular biology and general chemistry techniques used in evaluating the cell function through cytoskeletal and Sex Hormone Binding Globulin (SHBG/ABP) signaling changes, RT-PCR expression of different antioxidant agents and culture medium lactate concentrations, respectively.

2. Materials and methods

2.1. Cell culture and treatment

A Sertoli cell line from a mouse testis (42GPA9) was used (Bourdon et al., 1998). The cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (FBS), 1× non-essential amino acids, 2 mM L-glutamine, 5000 U/mL penicillin, and 5000 μg/mL streptomycin. FBS was heat-inactivated prior to use. Cells were plated on T25 flasks (2×10^6 cells) or on "flasks on slide" (Thermo Fisher Scientific Nunc,), cultured at 32 °C in a 5% CO_2 atmosphere and used at 80–90% confluence of cell monolayer. To simulate weightlessness, the cells were placed in a 3D Random Positioning Machine (RPM, DuthSpace, NL) and rotated (56°/s; 10^{-6} G) for 6, 24 and 48 h. Static controls (1×G) were treated in parallel and positioned on the supporting frame of the RPM in order for the cells to obtain the same vibration stress like those in modeled microgravity samples. At the end of the rotation, the cultured cells were fixed with 4% paraformaldehyde in PBS and then submitted for immunohistochemistry or used for RNA extraction. The culture medium of Sertoli cells which underwent microgravity or were used as control cells was utilized for lactate measurement.

2.2. Immunohistochemical analyses

The slides containing the cell cultures were removed from the flasks and underwent indirect immunofluores-

cence technique (Coons et al., 1955). After permeabilization with Triton X-100 (Sigma) 01% in PBS, PBS washing and exposure to Normal Goat Serum (diluted 1:50 in PBS; Sigma) in a humid chamber at 20 °C, the cells were incubated overnight at 4 °C with the antisera to α-tubulin (raised in mouse, diluted 1:500 in PBS, Sigma) or to-Sex Hormones Binding Globulin (SHBG/ABP, raised in rabbit against amino acids 197–403 mapping at the C-terminus of SHBG of mouse origin, diluted 1:100, Santa Cruz Biotechnology, Inc.). After PBS washing (0.01 M, pH 7.4), a second layer of fluorescein-isothiocyanate conjugated γ-globulins (FITC), goat anti-mouse (diluted 1:100 in PBS, Sigma), goat anti-rabbit (diluted 1:100 in PBS, Sigma) and mouse anti-goat (diluted 1:100, Santa Cruz Biotechnology, Inc.) was added for 30 min into a humid chamber, at 20 °C according to the specificity of the antisera. 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 non-immune rabbit serum or PBS. Immunoreactions were visualized using a conventional epifluorescence microscope.

2.3. RNA isolation and real-time RT-PCR

Total RNA was isolated by the acid phenol-chloroform procedure (Chomczynski and Sacchi, 1978) using the Trizol reagent (Sigma) according to the manufacturers' instructions. The purity of RNA was checked using absorption spectroscopy by measuring the 260/280 ratio. Only high purity samples ($OD_{260/280} > 1.8$) were subjected to further manipulation. The quality of isolated RNA was assessed by electrophoresis on 1.5% formaldehyde–agarose gel to verify the integrity of the 18S and 28S rRNA bands. First strand cDNA was synthesized from 1 μg of total RNA using 200 ng oligo(dT) 18-primer (TIB Mol Biol, Italia), 200 U RevertAid H-Minus M-MuLV reverse transcriptase (Fermentas, Hannover MD, USA), 40 U RNAsin and 1 mM dNTPs (Promega, Milan, Italy) in a final volume of 20 μl. The reaction was performed in a Master-cycler apparatus (Eppendorf, Milan Italy) at 42 °C for 1 h after an initial denaturation step at 70 °C for 5 min. The expression levels of genes were quantified in 96-well optical reaction by using a Chromo 4™ System real-time PCR apparatus (Biorad, Milan, Italy). Real-time PCR reactions were performed in quadruplicate in a final volume of 20 μl containing 10 ng cDNA, 10 μl of iTaq SYBR Green Supermix with ROX (Biorad), and 0.25 μM of each primer pair (TibMolBiol, Genoa, Italy). The one encoding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize the expression data. The accession numbers of the genes used in the study and the primer sequences are given in Table 1. The thermal protocol included an enzymatic activation step at 95 °C (3 min) and 40 cycles at 95 °C (15 s), 60 °C (30 s) and 72 °C (20 s). The melting curve of the PCR products (55–94 °C) was also recorded to check the reaction specificity. The relative gene expression of target genes, in comparison to the GAPDH reference gene, was conducted following the comparative C_T threshold method (18) using the Biorad software tool Genex-Gene Expression Macro™ (Vandesompele et al., 2002). The normalized data was then expressed in terms of the relative quantity of mRNA (fold induction) with respect to the control cells. The results are reported as the mean ± SD of three experiments in quadruplicate.

2.4. Analysis of lactate concentration

The evaluation of lactate concentration in culture medium was carried out by enzymatic determination at 540 nm using a lactate reagent (Trinity Biotech), as suggested by the manufacturer.

2.5. Statistical analysis

Statistical analysis was performed by using ANOVA, followed by Bonferroni ad hoc post test (INSTAT software, GraphPad Software, Inc., San Diego, CA 92130, USA).

3. Results

3.1. Effect of modeled microgravity on cytoskeleton and Sex Hormone Binding Globulin

After 6 h in modeled microgravity, Sertoli cells did not show relevant morphological differences as compared with the cells maintained at 1×G. The cytoskeleton, identified

Table 1
Names and accession numbers of the target genes are listed together with the sequences of the specific primer pairs.

Gene name	Accession number	Forward primer [5′–3′]	Reverse primer [5′–3′]
MT-1	NM_013602	CTGCTCCACCGGCGG	GCCCTGGGCACATTTGG
MT-2	NM_008630	TCCTGTGCCACAGATGGATC	GTCCGAAGCCTCTTTGCAGA
PARP-1	NM_013063	TGCAGTCACCCATGTTCGATGG	AGAGGAGGCTAAAGCCCTTG
p53	NM_030989	GGCTCCTCCCCAACATCTTAT C	TACCACCACGCTGTGCCGAAA A
GST	NM_013541	GTGCCCGGCCCAAGAT	TTGATGGGACGGTTCACATG
CAT	NM_009804	CCTGAGAGAGTGGTACATGC	CACTGCAAACCCACGAGGG
Mn-SOD	NM_013671	GGCTCCCGGCACAACACAGCC	CCTCGTGGTACTTCTCCTCGGTG
GAPDH	NM_008084	GACCCCTTCATTGACCTCAAC	CGCTCCTGGAAGATGGTGATGGG

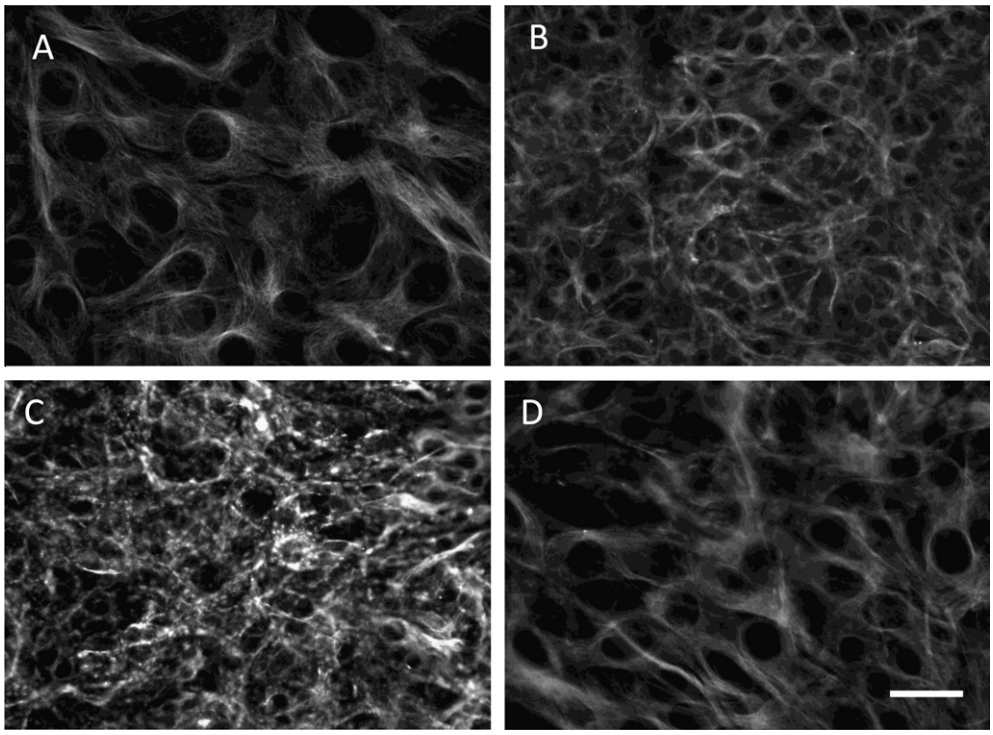

Fig. 1. Immunostaining of Sertoli cells for α-tubulin. Normal organization of microtubules was observed after 6 h in modeled microgravity (A). In cells undergoing simulated weightlessness for 24 h, the microtubules appeared disorganized and interrupted (B). A highly fragmented microtubular array was observed in cells after 48 h rotation (C). In control cells cytoskeletal organization was normal (D). Scale bars: 2.2 μm in (A); 4 μm in (B) and (C); 2.5 μm in (D).

with specific antibody directed against α-tubulin, was well organized, with microtubules radiating in discrete filaments from the nucleus to the plasma membrane (Fig. 1A). When the rotation was prolonged to 24 h, the microtubular array was extremely disorganized and appeared fragmented (Fig. 1B). The cytoskeletal alterations worsened with time: the interruptions in microtubules increased and, as a consequence, the cells lost their shape after 48 h of simulated weightlessness (Fig. 1C). In contrast, control (1×G) cells maintained their original cytoskeletal organization during the entire experiment (Fig. 1D).

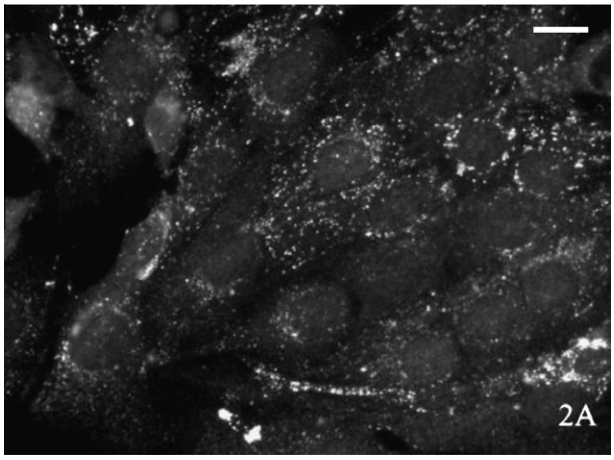

Fig. 2. Immunofluorescence for Sex Hormone Binding Globulin/Androgen-Binding Protein in control Sertoli cells. Scale bar: 2 μm.

An immunoreactive signal for Sex Hormone Binding Globulin (SHBG/ABP) was detected in the control cells, whereas, totally absent in cells maintained at 0×G (Fig. 2).

3.2. Effect of microgravity on lactate concentration

Lactate concentration in the culture medium was 2.44 ± 0.14 mM after 6 h of RPM treatment vs 2.5 ± 0.1 mM under control conditions. After 24 h, the values were 2.22 ± 0.01 vs 2.13 ± 0.11 mM in the RPM-treated and control cells, respectively. After 48 h of simulated weightlessness, values in treated cells (2.44 ± 0.095 mM) were slightly but significantly ($p \leqslant 0.01$) decreased with respect to controls (3.11 ± 0.3 mM) (Fig. 3).

3.3. Effect of microgravity on the antioxidants

In order to investigate the role of microgravity on the enzymatic constituents of the antioxidant system at the Sertoli cell level, SOD, CAT and GST activities of microgravity-exposed and control cells were evaluated. The pattern of MT expression during Sertoli cell exposure to microgravity was also investigated. Moreover, the expression of PARP-1, an enzyme normally activated by oxidative stress, and the protective role of the oncosuppressor gene p53 were also evaluated. After 6 h of rotation the expression of MT I, MT II, GST, CAT, SOD showed a remarkable increase ($p < 0.001$), PARP a significant decrease

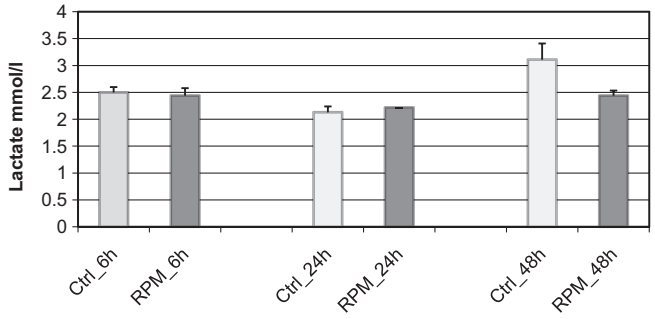

Fig. 3. Effects of simulated microgravity on *in vitro* lactate production by Sertoli cells. Lactate concentration was spectrophotometrically quantified in the culture medium of cells undergoing rotation or control conditions for 6, 24 and 48 h. Data is expressed as mean ± SD of $n = 3$ independent experiments. Differences among the means of microgravity and control cells were assessed using ANOVA test followed by Bonferroni ad hoc post test. *$p < 0.01$.

($p < 0.001$) with respect to controls, while p53 did not significantly change (Fig. 4A). When the experiment was prolonged to 24 h, all the investigated parameters showed an appreciable increase ($p < 0.001$) in the rotated samples (Fig. 4B). However, after 48 h of simulated microgravity the expression of all the analyzed enzymatic and non-hose enzymatic proteins were significantly lower ($p < 0.001$) than those of static controls (Fig. 4C).

4. Discussion

As mankind fully enters the Moon and Mars era, the prospect of humans to maintain a normal life cycle in space, including that of reproduction, becomes a critical problem. Reproduction experiments in microgravity have been carried out with insects, fish, amphibians, and birds, however, only a few studies concerned mammals. Spermatogenesis is a complex, highly ordered process of cell division and differentiation by which spermatogonia become mature spermatozoa. Within the seminiferous epithelium, Sertoli cells play a crucial role in the development of germ cells and in the regulation of spermatogenesis. These somatic cells, receive input from circulating endocrine effectors and paracrine factors from Leydig, peritubular and germ cells. These signals are integrated, thus allowing the secretion of products that control germ cell development and modulate the function of the other testicular cells, including their own.

In modeled microgravity the Sertoli cell line GPA9 showed similar damages to the cytoskeleton, as previously observed by us in the primary testicular cell line (BS PRC 57/Ste cells from trypsinized swine 1 month old testes) (Uva et al., 2007) and in other cell types such as: glial, endothelial, thyroid cells or lymphocytes (Uva et al., 2002; Sciola et al., 1999; Infanger et al., 2006; Cogoli-Greuter et al., 1994; Gmunder et al., 1990). This treatment also affected the expression of the SHBG/ABP in Sertoli cells. Such an effect might significantly impair the efficiency of spermatogenesis, since the carrier steroid SHBG/ABP is

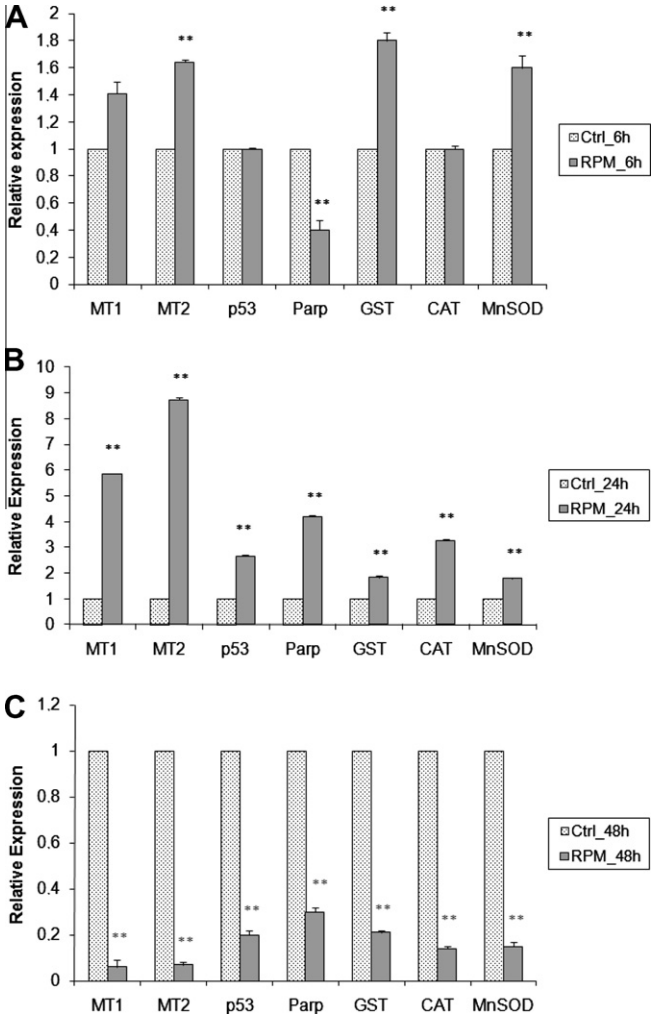

Fig. 4. Effects of simulated microgravity on the expression of antioxidant enzymes, p53 and PARP-1 by Sertoli cells. Relative expression of metallothioneins (MT-I and MT-II), catalase (CAT), superoxide dismutase (SOD), glutathione transferase (GST), p53 and PARP-1 were quantified in Sertoli cells after 6 h (A), 24 h (B) and 48 h (C) rotation by real-time RT-PCR. Values represent the fold induction as compared to the respective controls after normalization for GAPDH mRNA as a reference gene. Data are expressed as means ± SD of three independent experiments. Differences among means were assessed using ANOVA test followed by Bonferroni ad hoc post test. **$p < 0.001$ as compared to the respective controls.

essential in setting up and maintaining a special androgen environment required for normal germ cell differentiation. Our findings also demonstrate that microgravity, which is capable of disturbing Sertoli cell production of lactate, a preferential energy substrate for germ cells, could impair the nutritional function of Sertoli cells and consequently the spermatogenic process.

Regarding the risk of oxidative stress, the testes have developed a sophisticated array of antioxidant systems which includes both enzymatic and non-enzymatic constituents (Aitken and Roman, 2008). The present study suggests that simulated microgravity increases the level of oxidative stress in Sertoli cells determining initially a marked increase in the expression patterns of the major

ROS processing enzymes followed by a significant decrease later on, in agreement with recently reported data on microgravity-exposed rat PC12 cells (Wang et al., 2009). It is also tempting to speculate that the microgravity-induced oxidative stress in Sertoli cells could result in cellular senescence. In fact, oxidative stress is a major factor in the aging process (Jennings et al., 2000) and cellular senescence has been demonstrated to be associated with a rise in intracellular ROS when cells or organisms are exposed to the stress of a microgravity environment (Griswold, 1993; Li et al., 2004; Qu et al., 2006).

In addition our findings show that MT expression is up-regulated in microgravity-exposed cells during the initial phase and down-regulated afterwards. This suggests the existence of a possible interplay with ROS processing enzymes, which agrees with the fundamental role of MTs in refurbishing zinc to antioxidant zinc-dependent enzymes and proteins. Moreover, MTs could act as antioxidants themselves (Maret and Vallee, 1998).

Thus the overall antioxidative ability in Sertoli cells results in an increase at the beginning of microgravity but decreases subsequently. The initial increase could reflect a defensive response or adaptation to the microgravity stress at the beginning of the culture, followed by a dramatic decrease, suggesting the inability of antioxidant enzymes to prevent oxidative stress.

Furthermore, the relative expression of both p53 and PARP-1, which could represent a complementary and additional way by which Sertoli cells may face the noxious effects of ROS accumulation, revealed a similar pattern comparable to the changes observed in ROS processing enzymes and MTs.

Poly (ADP-ribose) polymerase is a zinc-finger DNA-binding enzyme which detects and signals DNA strand breaks generated either directly during base excision repair, or indirectly by genotoxic agents such as oxygen radicals. In response to genotoxic injury, PARP catalyzes the synthesis of poly (ADP-ribose), from its substrate β-NAD+. This polymer is then covalently bonded to several nuclear proteins and PARP as well. As a result, PARP converts DNA breaks into intracellular signals which activate DNA repair programs (Decker and Muller, 2002). In this regard, a similar function of PARP could be also assumed for Sertoli cells in response to microgravity-induced oxidative stress.

An up-regulation in p53 target genes is a conserved expression response to oxidative stress among different organs and species, including *in vitro* and *in vivo,* suggesting its important role in the induction of gene expression in response to oxidative stress (Han et al., 2008). Our results are in agreement with this hypothesis.

Altogether, our results indicate that Sertoli cells, when exposed to microgravity, express a reduced amount of proteins with antioxidant effects. These findings are in agreement with recent unpublished data on human stem cells that demonstrate a differential expression profile when using proteomic and genomic approaches, as well as, morphological alterations when cells are subjected to increased microgravity conditions (Blaber et al., 2009).

It is now acknowledged that antioxidant protection measures allow the testis to sustain its dual function of steroidogenesis and sperm production. The present results demonstrate that microgravity appears to disturb such defense system at the Sertoli cell level, thus generating a state of oxidative stress. Since Sertoli cells play an essential role in the control of spermatogenesis, it can be hypothesized that the observed changes exert a significant inhibitory effect on germ cell differentiation within the testes, which may result eventually in male infertility.

Today, it seems to be more and more relevant to also explore this topic in 0×G, with the perspective of possible Earth-bound applications. In fact, it has been more than 15 years that we have been interested in this field, ever since we demonstrated reduced androgen levels in male astronauts, a rather common condition known as "aging male syndrome" on Earth. As life expectancy increases, men are marrying later and have children in their 50s and over. Thus, male infertility has become also a social concern, often extending to those 60 years of age and older.

In conclusion, by using a Sertoli cell line, our data clearly shows that microgravity alters Sertoli cell function by affecting cytoskeleton, as well as its other well know functions such as ABP and lactate production. Our findings also demonstrate that microgravity disturbs antioxidant protection. In our opinion, further experiments need to be carried out in space in order to evaluate how much and through which mechanism microgravity affects both steroidogenesis and spermatogenesis, and therefore rule out possible risks of male infertility consequences both for future generations in space and the present generation here on Earth.

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