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Dorian Nowacki[2, A–D], Francesca G. Klinger[3, C–E], Grzegorz Mazur[1, E, F], Massimo De Felici[3, A, C–F]

Effect of Culture in Simulated Microgravity on the Development of Mouse Embryonic Testes*

[1] Department and Clinic of Internal and Occupational Diseases and Hypertension, Wroclaw Medical University, Poland
[2] Department of Human Nutrition, Wrocław University of Environmental and Life Sciences, Poland
[3] Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Rome, Italy

A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. All known organisms develop and evolve in the presence of gravitational force, and it is evident that gravity has a significant influence on organism physiology and development. Microgravity is known to affect gene expression, enzyme activity, cytoskeleton organization, mitotic proliferation and intracellular signaling.
Objectives. The aim of the present study was to study some aspects of the development *in vitro* of mouse embryonic testes in simulated microgravity.
Material and Methods. Testes from mouse embryos (12.5–16.5 days *post coitum*, d.p.c.) were cultured in simulated microgravity and standard static culture conditions. The microgravity condition was provided by a Rotary Cell Culture System (RWV) bioreactor, an apparatus designated for 3D tissue and small organ cultures. After 48 h of the culture in the RWV, testis morphology and size was evaluated.
Results. The first observation was that the culture in the RWV bioreactor had a beneficial effect on the testis growth and on the survival of germ cells in comparison to static 2D culture methods. Moreover, we found, that RWV culture caused disorganization the gonadal tissues, namely of the testis cords.
Conclusions. The results suggest that the maintenance of testis cord could be sensitive to microgravity. We hypothesize that while the effect on testis growth is due to a better nutrient and oxygen supply, the testis cord's disorganization might depend on the microgravity conditions simulated by the bioreactor. Considering the complexity of the processes involved in the formation of the testis cords and their dynamic changes during the embryo fetal period, further studies are needed to identify the causes of such effect (**Adv Clin Exp Med 2015, 24, 5, 769–774**).

Key words: rotary cell culture system, microgravity, embryonic testis, testis cords.

Cells and tissues grow, develop and interact in a highly complex 3D environment. Yet the methods of culturing and studying cells have traditionally been carried out in 2D on flat surfaces often composed of treated polystyrene or glass. A number of techniques now exist to establish 3D cell culture models that are better able to mimic the *in vivo* characteristics of cells and tissues in the body. In the present work, we experimented with the Rotating Wall Vessel (RWV) bioreactor as an *in vitro* 3D culture system for mouse embryonic testis.

The development of the testis during the embryonic and fetal periods is obviously crucial for male reproduction and defects in this process result in subfertility or even infertility in the adult. Researches on this process have largely focused on two key events such as sex testis determination and testis cord establishment. In the first of these events, the expression of *Sry* in the XY gonad

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triggers a signaling cascade, which establishes the Sertoli cell lineage and thus assures testis identity and the differentiation of other somatic cell lineages, namely Leydig cells. The second event relies upon expansion of the Sertoli cell population as well as the migration of cells from the mesonephros, namely the peritubular myoid cells. The remodeling and maintenance of the testis cords during the period between testis cord establishment and birth represents a third process that is crucial for the definitive organization of the seminiferous tubules in which spermatogenesis beginning at puberty will continue throughout the majority of the male's life [1–5].The unique microenvironment generated by RWV bioreactors should provide an excellent *in vitro* system for evaluating cell--cell and cell-matrix specific interactions, as well as for testing the influence that physical or chemical factors may have on organ histo-morphogenesis [6]. Devised by NASA's Johnson Space Center technological research in USA, the RWV bioreactor is a horizontally rotating, transparent clinostat with no internal moving parts, that leaves no head space between the atmosphere and culture medium, therefore reducing shear forces and turbulence normally associated with impeller-driven stirred bioreactors, to a minimum; sedimentation and inadequate gas/nutrient supply are also avoided, thus guaranteeing the most favorable conditions for cell/tissue culturing. Moreover, since the RWV uses the principle of clinorotation, that is, the cancellation of the force of gravity by rotation around one or two axes, it creates a microgravity environment in which the effect of such conditions on cell functions and morphogenesis of tissues and small or pieces of organs can be investigated on the ground [7–8].

Material and Methods

Collecting and Culture of Testes

The testes were dissected from mouse embryos obtained from pregnant CD-1 at the indicated age (12.5–16.5 days *post coitum*, d.p.c.). The mice were sacrificed by cervical dislocation following recommended procedures. Testes with the attached mesonephros were then transferred for culture in MEM (minimum essential medium; Invitrogen) supplemented with 2 mM L-glutamine (Sigma), 0.02 mL of Hepes (Sigma), 100 μg/mL penicillin (Sigma), 100 U/mL streptomycin (Sigma), 1 mg/mL bovine serum albumin (BSA; Sigma), 0.2mg/mL *N*-Acetyl-L-cysteine (Sigma), 0.36 mg/mL pyruvic acid (Sigma), and 10% fetal bovine serum (FBS; Gibco).

Three different culture conditions were used: 1) Culture on agar blocks (standard *in vitro* 2D culture). Testes were cultured on agar 3–4 mm cubes of 2% agar (dissolved in phosphate buffered saline, PBS) prepared in sterile conditions and left in the culture medium for 2–3 h at 37°C in a humidified incubator with 5% CO_2. Four blocks were transferred into a Petri dish (35 mm). The dish was filled with fresh culture medium up to the surface of the blocks. Testes were cultured (one per block) on the top of each cube; 2) Immersion culture. Testes were cultured onto a Petri dish pre-coated with agar to avoid attachment and completely immersed into the culture medium. This culture condition was employed as an additional 2D culture control since in the RWV, testes are completely immersed in the culture medium; 3) Culture in RWV. Testes were placed into a 10 mL RWV dish (not more than 5 testes in each dish). Rotary speed 14.7 rpm (rotations per min) was used. The obtained acceleration of gravity was calculated to be about 6.0 × mg instead of Earth's gravity 1 × g (Fig. 1). In all culture conditions, the testes were incubated for 48 h in a humidified incubator at 37°C with 5% CO_2 in air.

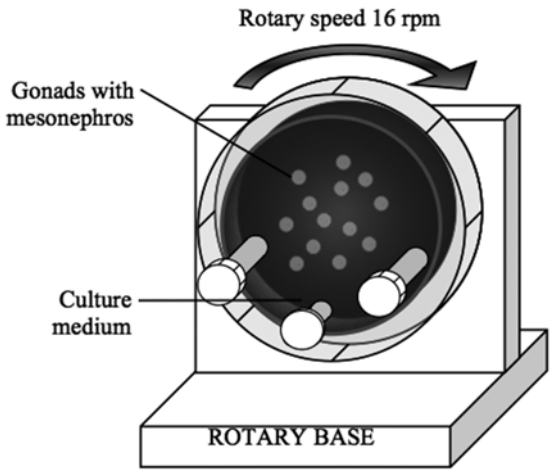

Fig. 1. Schematic representation of rotating wall vessel (RWV) bioreactor

Histology of the Gonads

Fixation

The testes were placed in Eppendorf tubes containing freshly prepared Bouin solution (71% picric acid, 24% formaldehyde and 5% acetic acid). After 2–3 h they were washed 3 times with 80% alcohol and stored in 70% alcohol at 4°C.

Tissue Processing

From 70% alcohol, the samples were transferred to 80% alcohol for 30 min and then in xylene (AlanaR) for the next 30 min. Finally, they were embedded in paraffin at 58°C. Samples were sectioned at 9–10 μm. The sections were placed in a warm water bath to reduce wrinkles and then transferred onto microscopic glass. Paraffin was removed from the samples using Histo-Clear (National Diagnostics), twice for 5 min. The samples were rehydrated and finally rinsed twice in water for 5 min before hematoxylin and eosin (HE) staining.

Testis Size and Cord Number Count

The length (L) and width (W) of each testis were measured under phase contrast microscope equipped with a micrometric ocular. Since a testis resembles an ellipsoid, the testis size was calculated using the ellipse area formula:

$$S = \pi \times (L/2) \times (W/2).$$

The number of cords in each testis was also counted.

Counting of Germ Cells

Longitudinal sections of the testis central part (at least 5 for each gonad analyzed in every treatment) were examined. Germ cells, identified on the basis of morphology, were counted in random squares of 2400 μm^2; only cells in which the nucleus was clearly visible were scored. Germ cells showing degenerative morphology (reduced size and highly condensed chromatin) were also scored.

Statistical Analysis

All statistical analyses are expressed as mean ± standard error (S.E.M.). For each culture group, the data was obtained from at least three independent experiments. Comparisons were made by one-way ANOVA, using ISTAT software, P values < 0.05 were considered as significant and $p < 0.001$ as highly significant.

Results

A summary of the measurements of testis size in the freshly collected 12.5–16.5 d.p.c. and 12.5 and 14.5 d.p.c. testis cultured for 2 days under different conditions are shown in Fig. 2. We observed a progressive increase in testis size *in vivo* from 12.5 to 16.5 d.p.c. paralleled by a significant decrease in the number of testis cords. Both 12.5 d.p.c. and 14.5 d.p.c. testes cultured in 2D immersed conditions showed frequent necrotic areas and a highly significant smaller size in comparison to the freshly collected 14.5 and 16.5 d.p.c. testes, respectively. On the other hand, the 12.5 d.p.c. and 14.5 d.p.c. testes cultured onto agar blocks (CAB testes), showed sizes slightly smaller than those of the comparable age, freshly collected 14.5 d.p.c. and 16.5 d.p.c. testes. Testes cultured in RWV reached intermediate size between those cultured under 2D immersed conditions and onto agar blocks. Differences were, however, statistically significant only in comparison to the 2D cultured immersed testes, thus indicating that while 2D CAB and immersed were the best and worse *in vitro* culture condition for testes growth, respectively, the culture in RWV allowed the testes to reach a size similar to the 2D CAB and *in vivo* conditions despite complete medium immersion.

As reported in previous studies, we observed that during the fetal period, testis cords undergo a substantial remodeling resulting in a reduction of their number between 12.5 and 16.5 d.p.c. We found that such changes were not significantly affected by either 2D culture conditions, but appeared severely affected in RWV, in which a significant reduction of the testis cord number occurred (Fig. 3).

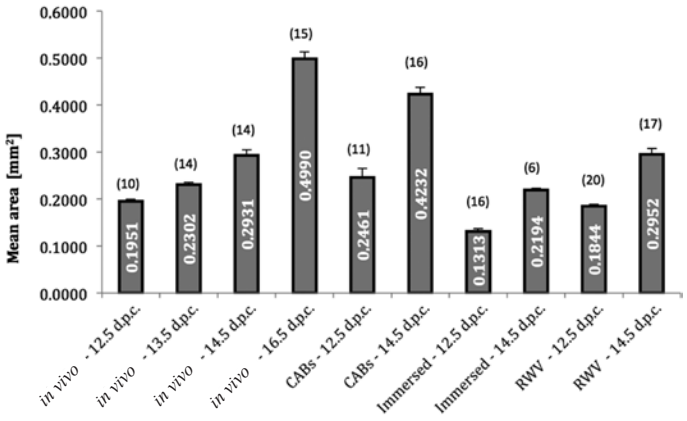

Fig. 2. Testis size (mean area ± S.E.M.) *in vivo* and under different 2-day *in vitro* conditions (in parentheses the number of the scored gonads)

CABs – testes cultured onto agar block.

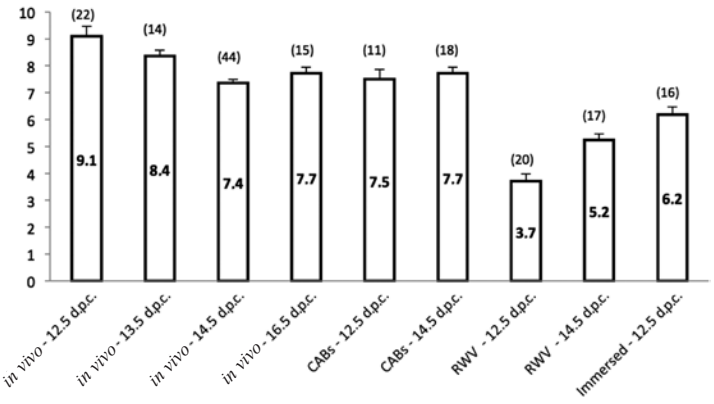

Fig. 3. Number of testis cords (mean ± S.E.M.) *in vivo* and under different 2-day *in vitro* conditions (in parentheses the number of the scored gonads). CABs – testes cultured onto agar block. Cord number of 14.5 d.p.c. testis cultured under immersed conditions was not evaluated because of extensive necrosis of tissue.

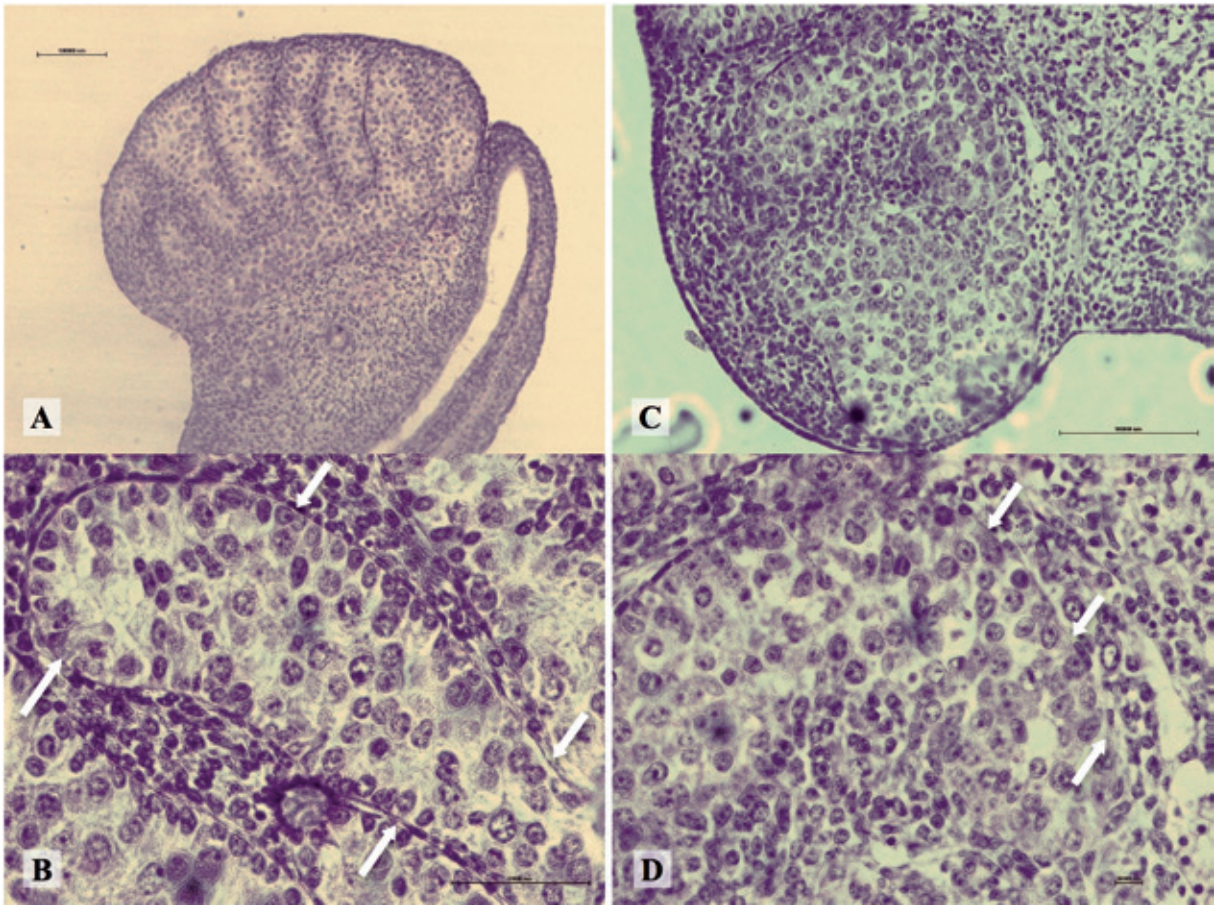

Fig. 4. Representative sections of male gonads. Testis cultured for 2 days under 2D CAB conditions showing normal cord structure: arrows indicate basal lamina (A, B). Sections of testes cultured for 2 days in RWV showing (arrows) disorganized cords and discontinuous basal lamina (C, D).

Histological analyses of the CAB 12.5 d.p.c. testes showed a well-preserved morphology resembling that of testes of comparable *in vivo* age (14.5 d.p.c.). The cords were well outlined and filled with germ cells, showing typical characteristics of prospermatogonia with relatively large, spherical nuclei and a few prominent nucleoli. A significant number of prospermatogonia showed coarse flakes of chromatin associated with the nuclear membrane typical for post-mitotic germ cells. Immature Sertoli cells were located at the periphery of the cords, whereas Leydig cells and other interstitial cells were located between the cords (Fig. 4). Sections of testes cultured under 2D immersion showed clear signs of tissue degeneration. In some sections, defined cords were difficult to identify; although the cords were well conserved in regions where the tissue appeared better preserved (not shown). In testis cultured in RWV, cord disorganization was evident, whereas the cell number and morphology did not appear grossly abnormal (Fig. 4). The mean number of prospermatogonia in the analyzed areas and their apparent normal or degenerating morphologies are

Table 1. Mean number + SEM of prospermatogonia in testis sections

	Mean number of cells		Mean number of cells – normal morphology		Mean number of cells – apoptotic morphology	
14.5 d.p.c. *in vivo*	10.91	± 0.367	9.82	± 0.387	1.09	± 0.113
CABs – 12.5 d.p.c.	10.04	± 0.269	8.88	± 0.255	1.16	± 0.118
Immersed – 12.5 d.p.c.	10.20	± 0.366	6.10	± 0.266	4.10	± 0.259
RWV – 12.5 d.p.c.	12.80	± 0.289	11.32	± 0.271	1.48	± 0.132

shown in Table 1. No significant alteration in germ cell number or increase in the number of degenerating cells were observed in testes cultured onto agar blocks or in RWV in comparison to the *in vivo* conditions. On the contrary, in testes cultured in 2D immersed conditions, a significant reduction in the number of prospermatogonia and increasing numbers of cells showing degenerating morphologies in comparison to the *in vivo* and the other *in vitro* conditions were found.

Discussion

The RWV was designed to provide continuous sedimentation of particles through a culture medium, which is rotating essentially as a solid mass with minimal induced cellular shear and turbulence. RWV largely solves the challenges of suspension culture: to suspend cells and small organs without inducing turbulence or large degrees of shear, while providing adequate nutrition and oxygenation. RWV has been successful in culturing prostate organoids, liver, colon carcinoma and cartilage, among other tissues [10].

In the present paper, for the first time, we used the RWV bioreactor for *in vitro* culturing of mouse fetal testes during a crucial period of their morphogenesis from 12.5 d.p.c. to 16.5 d.p.c. In the mouse embryo, the sex differentiation of the gonadal anlage begins around 10.5–11.5 d.p.c., when *Sry* activation leads to Sertoli cell differentiation. Soon after, testis cords develop and are visible by 12.5 d.p.c. under a dissecting microscope; the cords consist of Sertoli cells surrounding the germ cells and separate them from the mesenchymal interstitium [11]. During the subsequent and up to the perinatal period, testes undergo a considerable size increase paralleled by cell differentiation and very dynamic cord changes [5, 12]. Sertoli cell proliferation, migration of mesenchymal cells into the testis and congregation of interstitial cells around the forming cords, are thought to be the main causes for the increase in testis size [12]. Our results suggest that, in testes cultured both under RWV and standard 2D onto agar block conditions, these processes are likely to be well maintained. Actually, throughout the culture period we observed a size increase in cultured testes analogous to *in vivo*. At the same time, the cell morphology and germ cell number resembled that of testes of comparable *in vivo* age. In this regard, it is worth noting that the culture in RWV was able to markedly alleviate the adverse effects of static 2D immersed conditions on testis development, likely providing better cell nutrition and oxygenation.

Certainly, however, the more intriguing effect of RWV culture conditions which we observed was the striking disorganization of the testis cords. Previous studies have shown that the expression of several genes, such as *Inba*, *Wt1*, *Sox9* and *Sox8* in Sertoli and Leydig cells, are necessary for the maintenance of the testis cords after their initial formation [13–15]. Unpaired expression of these genes could result in defects in molecular signaling controlling the correct differentiation and activity of testicular cells. This in turn might downregulate basal lamina components, which results in a breakdown of the basal lamina and testicular cord disorganization. In fact, interaction between Sertoli cells and peritubular myoid cells leads to the polarized secretion of various extracellular matrix (ECM) proteins, such as laminin, type IV and IXa3 collagen and heparan sulfate. These ECM molecules are required for formation of the basal lamina, which surrounds the testis cords and maintains their structural integrity [16–18]. Our morphological observations indicate that in testes cultured in RWV, the basal lamina surrounding the cords was intermittently disrupted in comparison to the continuous lamina in the control. Thus, the question arises as to which mechanism is responsible for the loss of testis cord basal lamina seen in RWV culture. As the RWV was developed by NASA to simulate culture conditions predicted to occur during experiments in space, these conditions were defined as simulated microgravity. We hypothesize that such microgravity conditions could be responsible for altered production of basal lamina molecules and/or of their assembly. Although it is known that the altered mechanical culture conditions of spaceflight might

have dramatic effects on cell growth and metabolism, as well as the production of bioproducts from growth hormones to hybridomas [10], the mechanisms by which mechanical culture conditions, including microgravity, could affect gene expression, protein synthesis and the structure and function of diverse cell types is only beginning to be investigated. Postulated mechanisms include cytoskeletal changes, electrical and chemical signaling and channel activation [10]. The results presented in the present paper offer the possibility to investigate such mechanisms in the model of fetal testis development that is also relevant for future reproduction in space.

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Address for correspondence:

Dorian Nowacki
Department of Human Nutrition
Wrocław University of Environmental and Life Sciences
Faculty of Food Science
Chełmońskiego 37/41
51-630 Wrocław
Poland
E-mail: dorian.nowacki@wp.pl

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