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Functional *in vitro* model to examine cancer therapy cytotoxicity in maturing rat testis

Mirja Nurmio[a,*], Jorma Toppari[a,b], Jenny Kallio[a], Mi Hou[c], Olle Söder[c], Kirsi Jahnukainen[c,d]

[a] *Department of Physiology, University of Turku, FIN-20520 Turku, Finland*
[b] *Department of Pediatrics, University of Turku, FIN-20520 Turku, Finland*
[c] *Pediatric Endocrinology Unit, Department of Woman and Child Health, Karolinska Institute and University Hospital, SE-171 76 Stockholm, Sweden*
[d] *Division of Hematology-Oncology and Stem, Cell Transplantation, Hospital for Children and Adolescents, PO Box 281, FIN-00029 Helsinki, Finland*

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ABSTRACT

Childhood cancer treatment can lead to infertility. Organ culture of early postnatal testicular tissue might provide a valuable approach to the study of acute testicular toxicity. The aim of the present study was to develop a functional *in vitro* organ culture method, in order to identify sensitive target cells to doxorubicin-induced cytotoxicity in immature rat testis during germ cell migration prior initiation of the first wave of spermatogenesis. Testicular tissue fragments from 5-day-old Sprague–Dawley rats were cultured in the absence or presence of doxorubicin (40 and 100 ng/ml) and morphology, apoptosis, proliferation and testosterone secretion was analyzed. Postnatal testicular development proceeded normally in control samples for 48 h *in vitro*. In these untreated culture conditions germ and Sertoli cell numbers and germ cell migration were comparable to *in vivo*. Germ cells were the primary, most sensitive targets for *in vitro*-induced doxorubicin (100 ng/ml) toxicity and their death was not associated with any morphological defects in the Sertoli cells. Organ culture which reduces the need of animal experimentation can be used to study the cytotoxic effects of doxorubicin on the immature testis.

1. Introduction

Early postnatal life is a critical period in the development of male reproductive functions essential for adult fertility. During this period the pool of spermatogonial stem cells and their niches are being formed and exhibit particularly pronounced sensitivity to deleterious effects of several cytotoxic agents [1,2].

For decades, organ cultures of the postnatal testis have been employed to investigate testicular development, and initiation of the first wave of spermatogenesis occurs in such cultures [3–6]. The effects of hormones, growth factors [6,7], environmental pollutants [8] and irradiation [9] on early postnatal maturation of the testis have been studied with this approach. Organ cultures of immature testicular tissue might provide a valuable system to study short-term toxic effects of chemotherapeutic agents. This is important since, treatment of cancer in young boys with these agents has long been known to result infertility problems. However, in clinical studies it is difficult to monitor short-term toxic effects of such treatments.

Fragments of both fetal and neonatal human and rodent testicular tissue can be maintained *in vitro* for at least 3 days without loss of the structural integrity of the seminiferous cords. However, several investigators have observed cell death in the central regions of larger fragments of postnatal testicular tissue (0.5–1 mm^3) after 3 days in culture [6,10]. Furthermore, in the case of testicular tissue from the juvenile rhesus monkey severe disintegration of tubular morphology occurs after only 48 h in culture [9].

The aim of present study was to develop a functional *in vitro* organ culture method, in order to identify sensitive target cells of doxorubicin toxicity in immature rat testis during germ cell migration prior to initiation of the first wave of spermatogenesis. The possibility of reducing animal experimentation by use of this organ culture system was also evaluated.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats were housed at the Animal Center of Turku University (Turku, Finland) in a controlled environment with access to food and water *ad libitum*. All experiments were pre-approved by the Committee on the Ethics of Animal Experimentation at University of Turku. The day of birth was designated as day 0. Caused by laboratory routines variation in detection of birth of pups was maximally 10 h.

* Corresponding author. Tel.: +358 23337373; fax: +358 22502610.
E-mail address: mirja.nurmio@utu.fi (M. Nurmio).

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In vitro: in each study measurements were made on tissue from at least four different rat pups at each time-point and dose employed. Since, one 5-day-old animal provided maximally 10 testicular fragments for culture, material from one animal were used to perform cultures of different doses and time-points. *In vivo*: four control animals per each time-point were used to investigate the physiological incorporation of ^{3}H-thymidine and BrdU in testicular tissue. No doxorubicin exposures were done *in vivo* in the present study.

2.2. Preparation and treatment of organ cultures

Following decapsulation, the testes of 5-day-old rat pups were dissected into pieces 1 mm^3 in size, which were then placed on filters (Millipore, ISO-PORE, Membrane Filters, Carrigtwohill, Ireland) immersed in 1 ml of Dulbecco's Modified Eagle's Medium/F12 medium (1:1) (DMEM/F12) supplemented with 0.1% BSA and gentamycin (50 μg/ml), maintained at +34 °C and equilibrated with an atmosphere containing 5% CO_2. The filters with these tissue fragments were placed in sterile, four-well plastic culture plates (NUNC™ Brand Products, Denmark) in an incubator (at 34 °C, and under 5% CO_2) in the presence or absence of doxorubicin (Pharmacia Upjohn, Stockholm, Sweden) (0, 40 or 100 ng/ml) for 6, 12, 16, 24, 48 or 72 h.

In vitro concentrations were selected to correspond to the serum measurements of our previous *in vivo* study [2] and serum concentrations detected in clinical pharmacokinetical studies [11]. Single dose of 3 mg/kg of doxorubicin used to 6-day-old rats (corresponding calculated rat surface related dose of 6.7 mg/m^2) has been shown to lead to mean peak serum concentration of 190 ng/ml 20 min after injection [2]. In clinical study single doxorubicin dose of 40 mg/m^2 (corresponding the calculated human weight related dose of 1.3 mg/kg) has been shown to lead to mean peak serum concentrations of 50–70 ng/ml [11]. It has to be considered, however, that under *in vivo* situation the length of the maximum plasma concentration of the drug may vary compared to steady state exposure *in vitro*.

2.3. Monitoring cell proliferation as incorporation of ^{3}H-thymidine into DNA

During the final 4 h of the culture period, proliferating testicular cells were exposed to 0.5 μCi ^{3}H-thymidine (specific radioactivity 5.0 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK). Subsequently, the labelled tissue samples were gathered with a Skatron Instruments Cell Harvester (Skatron, Lier, Norway), placed on a glass fiber filter and rinsed with distilled water. Discs were punched out of these filters (which contained high-molecular weight material, including DNA) and put into small plastic tubes. Three milliliters of Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA) were added to each tube and the radioactivity (cpm) was measured by a Beckman LS5000CE scintillation spectrometer.

In order to examine physiological testicular incorporation of ^{3}H-thymidine in control rats *in vivo*, 5-, 6- and 7-day-old rats were injected i.p. with this substance (1.0 μCi/g body weight) 4 h prior to sacrifice and their testicular tissue then processed in a similar manner to the *in vitro* samples.

2.4. Immunohistochemical detection of BrdU, WT-1 and cleaved caspase-3

BrdU (final concentration, 100 μM; Roche Applied Science, Indianapolis, USA) was added to the medium of the testicular organ cultures 1 h before termination of the incubation period. In the case of detecting proliferating cells *in vivo*, BrdU was injected i.p. 3 h prior sacrifice of the animal (50 mg/kg). Thereafter, the tissue fragments were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 4-μm thick sections. After dewaxing and rehydrating, incorporation of BrdU by these sections was determined immunohistochemically in accordance with the manufacturer's instructions (Roche Diagnostics Corp., Indianapolis, USA), as described earlier [12].

Dual immunohistochemistry was performed as described earlier [9], with minor modifications. In brief, following treatment with citrate buffer, the slides were incubated in 2N HCl at 37 °C for 30 min, then neutralized in 0.1 M borate buffer for 10 min and finally placed in 0.85% NaCl for 3 min. For dual immunostaining, a rabbit polyclonal antibody directed against WT1 (180) (1:100 dilution; Santa Cruz, CA, USA) was used to identify Sertoli cells and a mouse monoclonal anti-BrdU antibody (6 μl/100 μl TBS Roche Diagnostics Corp.) to label proliferating cells.

Other slides were incubated with antibodies towards cleaved caspase-3 (Cell Signaling Technology Inc., MA, USA) or GATA-1 (1:100 dilution in both cases; Santa Cruz, CA, USA) subsequently labelled with the PowerVision immunohistochemistry kit (PowerVision+™ Poly-HRP IHC Kit Biotin-free, anti-mouse/rabbit, ImmunoVision Technologies Co., MA, USA) as instructed by the manufacturer and finally examined under a light microscope.

2.5. Determination of the apoptotic index by in situ analysis of oligo ligation

Apoptotic cells were detected with the ApopTag *in situ* oligo ligation (ISOL) kit involving oligo A, in accordance with the manufacturer's instructions (Chemicon International, Serologicals, Norcross, GA, USA). Briefly, following routine deparaffinization and rehydration, endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2 for 5 min. Next, the sections were washed with PBS and then briefly with dH_2O and thereafter digested with proteinase K (50 μg/ml in PBS) for 15 min. After washing again with dH_2O, the slides were treated with equilibration buffer for 30 s, after which they were washed once again and incubated with DNA ligase for 16 h in a humified chamber at 16–22 °C. After treatment with the streptavidin-peroxidase conjugate for 30 min, at room temperature, the sections were finally visualized with DAB. The morphological characteristics employed to identify apoptotic cells included condensation and localization of the chromatin at the nuclear periphery, nuclear disintegration, and diminished cellular size.

2.6. Electron microscopic analysis of the ultramorphology of the Sertoli cells

Testicular tissue fragments cultured *in vitro* were fixed by immersion in 5% glutaraldehyde dissolved in an s-collidine buffer (0.16 mol/L, pH 7.4) at 20 °C. Postfixation was achieved with 1% osmium tetroxide in 1.5% aqueous potassium ferrocyanide and the samples were then embedded in epoxy resin (Glycidether 100, Merck, Darmstadt, Germany). Ultrathin (70 nm) sections were subsequently prepared using an Ultracut E ultramicrotome (Reichert Jung, Vienna, Austria), stained with uranyl acetate and lead citrate, and finally examined under a JEOL 100SX electron microscope (JEOL, Tokyo, Japan).

2.7. Determination of the numbers of germ and Sertoli cells by light microscopy

Semi-thin (1 μm) sections prepared from testicular tissue embedded in epoxy resin as described above were stained with toluidine blue and then examined with oil immersion under a light microscope. The numbers of germ and Sertoli cells per cross-section of seminiferous cord were counted on the basis of their typical nuclear morphologies [13]. The location of the germ cells within the seminiferous tubule was also noted.

2.8. Testosterone assay

Testosterone secreted into culture medium was measured by radioimmunoassay, as described earlier [14]. The lower limit of detection employing this assay was 1 fmol/tube and the intra- and interassay coefficients of variation were <6% and <12%, respectively.

2.9. Presentation of the data and statistical analyses

In the case of incorporation of ^{3}H-thymidine, necrotis areas could not be separated from analysis, however, in all other studies areas of tissue containing necrotic cells were excluded. An analysis of ISOL was carried out counting the number of positive cells per 1 mm^2 of one section from a single cultured testicular fragment from each animal in each experimental group following 12, 16 and 24 h in culture. For the determination of other morphological, immunohistochemical and proliferative data, the germ, Sertoli and peritubular myoid cells in at least 40 round cross-sections of cords from each testis at each time-point and for each treatment were counted. The testosterone concentration was assayed in the media from four wells in each group following 24 h in culture.

The values obtained in the independent experiments were pooled for calculation of the mean values and S.E.M. The Mann–Whitney rank sum test or *t*-test was employed for single statistical comparisons of independent groups of samples. The results were analyzed for statistically significant differences using ANOVA, followed by the Tukey test for multiple comparison of independent groups of samples. A *p*-value of less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Morphogenesis of the postnatal rat testis in vitro

When fragments of testicular tissue from 5-day-old rat pups were cultured no signs of cell death or morphological disorganization were observed after 6 or 12 h. Following more than 24 h of organ culture, some of the biggest tissue samples began to lose tissue integrity in the middle region. However, most of the cultured samples maintained normal tissue morphology throughout the sample (Fig. 1). After 48 h in culture the central region of the tissue had lost its morphological integrity in all samples and the surrounding seminiferous tubules stained positively for cleaved caspase-3. A clear division between the intact and necrotic regions of the tissue was observed (Fig. 1). After 72 h in culture, necrosis was present in the outer layers of the samples as well. Accordingly, in the subsequent investigations only the intact regions of samples cultured for 24 or 48 h were subjected to morphological and immunohistochemical analyses.