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Time- and dose-dependent effects of ethanol on mouse embryonic stem cells

Sarah L. Worley, Brittney J. Vaughn, Alexander I. Terry, Catherine S. Gardiner, Gregory K. DeKrey *

School of Biological Sciences, University of Northern Colorado, 501 20th Street, Greeley, CO 80639, USA

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ABSTRACT

Ethanol is a common solvent used with mouse embryonic stem (mES) cells in protocols to test chemicals for evidence of developmental toxicity. In this study, dose–response relationships for ethanol toxicity in mES cells were examined. For cells maintained in an undifferentiated state, ethanol significantly reduced viable cell numbers with estimated half maximal inhibitory concentrations of 1.5% and 0.8% ethanol after 24 and 48 h, respectively, observations which correlated with significantly increased expression of apoptotic markers. For cells cultured to induce cardiomyocyte formation, up to 0.5% ethanol during the first two days failed to alter the outcome of differentiation, whereas 0.3% ethanol for 11 days significantly reduced the fraction of cultures containing contracting areas, an observation that correlated with significantly reduced cell numbers. These results suggest that ethanol is not an inert solvent at concentrations that might be used for developmental toxicity testing.

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1. Introduction

Embryonic stem (ES) cells are self-renewing, pluripotent cells isolated from the inner cell mass of blastocyst stage preimplantation embryos. After implantation, these cells largely develop into tissues of the fetus. As such, ES cells can be used as surrogates for studying the potential toxicity of drugs and other chemicals on cells of the inner cell mass or later developmental stages of those cells. The embryonic stem cell test (EST) was developed for this purpose and is now a validated in vitro protocol used as an alternative to embryotoxicity testing [1,2]. In part, this protocol examines the efficiency of mouse ES (mES) cell differentiation into cardiomyocytes as a predictor of toxicity.

Pluripotency of mES cells can be maintained by culturing them in the presence of serum plus leukemia inhibiting factor (LIF) [3,4]. Commonly used markers of pluripotency include alkaline phosphatase, stage specific embryonic antigen 1 (SSEA-1), nanog homeobox, and octamer-binding transcription factor 3/4 (Oct 3/4), all of which are expressed at higher levels by undifferentiated mES cells, and their expression declines within a few days once differentiation has begun [5–7]. In the absence of LIF, directed differentiation of mES cells can be achieved, as in the EST, by first initiating embryoid body formation using various culture techniques such as the hanging drop method (first described by Wobus et al. [8]) followed by adherent culture. In the EST the appearance of rhythmically contracting cells after approximately seven days indicates the presence of cardiomyocytes.

Ethanol is one of the solvents used in the EST to dissolve test chemicals that have limited water solubility. A recently recommended maximum final concentration for ethanol in the EST was 0.5% [1]. Use of ethanol at approximately this concentration for undifferentiated mES cells is supported by the findings of Arzumnayan et al. during 2009 who showed no decline in cell number or change in apoptotic markers after 48 h of exposure to ethanol at 100 mM (approximately 0.58%) [9]. However, mES cells that are cultured in the absence of LIF and have begun differentiating may have greater sensitivity to ethanol toxicity than their undifferentiated counterparts. For example, both Huang et al. in 2007 and Arzumnayan et al. in 2009 found enhanced apoptosis in differentiating mES cells after exposure to ethanol at 0.58% (v/v) or higher concentrations [9,10]. In addition, ethanol exposure may delay some aspects of mES cell differentiation. Adler et al. in 2006 have shown that the normal decline in Oct 3/4 expression by differentiating mES cells is delayed by ethanol exposure at concentrations as low as 0.25% [11]. These results suggest that ethanol at a concentration

* Corresponding author. Tel.: +1 970 351 2493; fax: +1 970 351 2335.
E-mail addresses: sworleyjones@gmail.com (S.L. Worley), brittney.j.vaughn@gmail.edu (B.J. Vaughn), terry.alex@rocketmail.com (A.I. Terry), catherine.gardiner@unco.edu (C.S. Gardiner), gregory.dekrey@unco.edu (G.K. DeKrey).

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of 0.5% may not be an inert vehicle for other chemicals when used on differentiating mES cells.

In this study, the toxicity of ethanol in mES cells was examined over a range of concentrations to further describe the dose–response relationships for two endpoint categories: (1) cytotoxicity and apoptosis in undifferentiated cells after short-term exposures (up to 48 h) and (2) differentiation into cardiomyocytes with concurrent exposures for two or eleven days. For cells maintained in an undifferentiated state, we show that ethanol exposure significantly reduced viable cell numbers with estimated half maximal inhibitory concentrations (IC_{50}) of 1.5% and 0.8% ethanol after 24 and 48 h, respectively, and loss of viable cells correlated with significantly increased expression of apoptotic markers. For cells cultured to induce cardiomyocyte formation, ethanol exposure at concentrations up to 0.5% during the first two days failed to significantly alter the outcome of differentiation, whereas 11 days of ethanol exposure (as in the EST) at concentrations as low as 0.3% significantly reduced the fraction of cultures containing contracting areas, an observation that correlated with significantly reduced cell numbers. These results suggest that ethanol is not an inert solvent at the highest concentration that might be used in the EST.

2. Materials and methods

2.1. Cell culture

Mouse ES D3 cells (strain 129S2/SvPas, ATCC, Manassas, VA, USA) or mES J1 cells (strain 129S4/SvJae, ATCC) were cultured in ES medium at 37 °C in air plus 5% CO_2 using the basic methods of Hill and Wurst [12]. ES medium was composed of Dulbecco's Modified Eagle Medium (catalog #11965118, Invitrogen, Carlsbad, CA, USA) supplemented with 12.5% ES qualified fetal bovine serum (Millipore, Billerica, MA, USA), 0.83 mM nucleosides mix (Millipore), 83 U/mL penicillin, 83 μg/mL streptomycin, 0.83 mM non-essential amino acids, 1.66 mM L-glutamate, and 0.1 mM β-mercaptoethanol. To maintain mES cells in an undifferentiated state they were cultured on mouse CF-1® embryonic fibroblasts in medium supplemented with 1000 U/mL leukemia inhibitory factor (LIF, Millipore). Embryonic fibroblasts were isolated and inactivated with mitomycin C as described by Connor [13]. mES cells were passed every other day and medium was changed every day. The undifferentiated status of mES cells was verified by the expression of alkaline phosphatase. All cells were detached from culture dishes using trypsin/EDTA except when they were to be analyzed by flow cytometry, in which case they were detached using TripLExpress (Invitrogen). Trypan blue exclusion was considered a measure of viability when counting cells microscopically.

2.2. Exposure to ethanol

Absolute ethanol was added to ES medium to achieve desired concentrations up to 3% (v/v, volume fraction), and these media were used to fully replace the media on all cells at the initiation of each experiment and at each successive media replacement. Unless otherwise indicated, within each experimental trial, the concentration of ethanol for each treatment group remained the same throughout the duration of the experiment. In experiments of 48 h duration, all mES cell cultures were initiated simultaneously (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, or 3.0% ethanol) and layered with mineral oil to minimize ethanol evaporation. Some cultures were then harvested at each time point: 8, 24, and 48 h. In experiments of 10 or 11 days duration, all mES cell cultures were initiated simultaneously and sealed inside Tupperware containers, to minimize ethanol evaporation, which also contained 300 mL of water containing ethanol at the same concentration as in the culture medium. Cytotoxicity experiments used ethanol concentrations of 0%, 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, or 1.5%. Differentiation experiments used ethanol concentrations of 0%, 0.1%, 0.2%, 0.3%, 0.4%, or 0.5%. Examination, analysis, and/or harvesting of each culture was performed at the termination of each experiment.

2.3. Cytotoxicity assays

Five different cell populations (each composed of a different cell passage) of mES D3 cells were cultured in ES medium (with LIF) in 96-well tissue culture plates, 12 replicate wells per population, and five cell populations per plate. Each plate constituted a different ethanol exposure group. After ten days in culture, a cytotoxicity assay was performed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described by Mosmann [14]. Formazan concentrations were detected using a plate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA).

2.4. Gene expression

The expressions of *Oct-3/4* and *Nanog* were used as markers for differentiation of mES D3 cells [5,6,15]. Total RNA was isolated from cells and cDNA was synthesized as previously described [16]. PerfeCta SYBR Green Supermix for iQ (Quanta Biosciences, Gaithersburg, MD, USA) was used for PCRs which were conducted using a MyIQ Single Color Real Time PCR Detection System (Biorad, Hercules, CA, USA). Reactions were carried out for 40 cycles with denaturing at 94 °C for 45 s, and elongation at 72 °C for 1 min. Official symbols (and alternate names where appropriate), NCBI GeneID numbers, annealing temperatures, and 5′–3′ primer sequences were as follows: *Nanog*, 71950, 63 °C, GCA-AGC-GGT-GGC-AGA-AAA-A, CAG-AAA-GTC-CTC-CCC-GAA-GTT-ATG; *Pou5f1* (*Oct-3/4*), 18999, 63 °C, AAT-GCC-GTG-AAG-TTG-GAG-AAG-GT, TGG-GGG-CAG-AGG-AAA-GGA-TAC; *Gapdh*, 14433, 63 °C, ACG-TGC-CGC-CTG-GAG-AAA, GGG-GGC-CGA-GTT-GGG-ATA-G. Quantification cycles (threshold cycles) for genes of interest were normalized to the quantification cycles for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, the reference gene) from the same samples. Reactions for reference genes and genes of interest were run concurrently.

2.5. Flow cytometry

All samples were analyzed using an Accuri C6 flow cytometer and CFlow Plus software, version 1. For detection of apoptosis, single-cell suspensions of cells were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V (Annexin) and propidium iodide (PI) according to the manufacturer's protocol (BD Pharmingen, San Jose, CA, USA). A minimum of 100,000 light-scatter-gated events were collected for each sample. Regions and quadrants were set based on negative (untreated) and positive (UV-irradiated) control cells.

For the detection of intracellular myosin heavy chain (MHC), single-cell suspensions of cells were stained with mouse anti-MHC antibody (clone MF20, Developmental Studies Hybridoma Bank) using the protocol developed by Seiler et al. [17]. Biotin-conjugated goat anti-mouse IgG secondary antibody (Rockland, Gilbertsville, PA, USA) was used with phycoerythrin (PE)-conjugated streptavidin (Rockland). A minimum of 1000 light-scatter-gated events were collected per sample. Regions were set for fluoresence data (FL2) based on negative control cells stained with secondary reagents only.

2.6. Differentiation into cardiomyocytes

Cardiomyocytes were generated through the hanging drop method as described previously [8,18]. Briefly, on day 0, hanging

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