



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



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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 Arzumayan 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 Arzumayan 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

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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% CO₂ 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 (Bio-rad, 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 fluorescence 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

drop cultures (20 μ L) were established with 500 undifferentiated mES D3 cells or 2000 undifferentiated mES J1 cells in ES medium (without LIF). On day 2, each embryoid body was transferred to an individual well of an ultra low attachment 96-well plate (Costar, catalog #3474) with fresh medium. On day 5, each embryoid body was transferred to a separate gelatinized (0.2%) well of a 48-well tissue culture cluster with fresh medium and incubated for up to six days. Thereafter, each well was examined under a microscope to visualize contracting areas which indicated the presence of cardiomyocytes.

2.7. Statistics

Statistical analyses were performed using the GLM and REG procedures of SAS for Windows version 9.1 (SAS Institute Inc., Cary, NC, USA). Two-way analysis of variance (ANOVA) was used for data sets containing experimental trial and ethanol concentration as sources of variance. Three-way ANOVA was used for data sets containing experimental trial, ethanol concentration, and cell passage number as sources of variance. Post-hoc all-pairwise *t*-tests were used to analyze significant differences between ethanol concentrations with no lower stringency than Fisher LSD. Differences between means were considered significant at $p \leq 0.05$. Regression analysis of dose–response trends was performed using the least-squares method, and slopes were considered significantly different from zero at $p \leq 0.05$.

3. Results

3.1. Effects of ethanol on undifferentiated mES cells

3.1.1. Ethanol exposure up to 48 h reduces undifferentiated mES cell numbers

To assess ethanol's impact on mES cell numbers, undifferentiated mES cells were cultured with or without ethanol for up to 48 h (Fig. 1). After 8 h of culture, relative to controls, no significant changes in cell numbers were observed at any ethanol concentration up to a maximum of 3% (data not shown). By contrast, after 24 h of ethanol exposure, a statistically significant and dose-dependent decrease in cell numbers was observed (Fig. 1A). A significant 42% decrease in cell numbers was observed at 1% ethanol. Higher concentrations of ethanol led to greater reduction of cell numbers up to 96% loss at 3% ethanol. An IC_{50} of 1.4% ethanol was estimated using the regression line [$y = (-0.32 \times 10^6)(x) + (1.52 \times 10^6)$] for the dose–response trend, the slope of which was significantly different from zero ($p < 0.001$). A statistically significant and dose-dependent decrease in cell numbers was also observed after 48 h of ethanol exposure (Fig. 1B). A significant decrease in cell numbers, by 91%, was first observed at 1.5% ethanol. The maximum decrease in cell numbers (by >99%) was observed at 3% ethanol. An IC_{50} of 0.8% ethanol was estimated using the regression line [$y = (-0.58 \times 10^6)(x) + (0.97 \times 10^6)$] for the dose–response trend, the slope of which was significantly different from zero ($p = 0.04$).

3.1.2. Ethanol exposure up to 48 h causes apoptosis in undifferentiated mES cells

To explore the cause of decreased mES cell numbers following ethanol exposure, undifferentiated mES cells were cultured with or without ethanol for up to 48 h and then analyzed by flow cytometry to detect apoptotic cells. As shown in Fig. 2, relative to controls, ethanol exposure caused a statistically significant dose- and time-dependent increase in the number of apoptotic cells. After 8 h of culture (Fig. 2A), a significant 20% decrease in viable (unstained) cells was observed at 2% ethanol which was accompanied by a significant 2-fold increase in early apoptotic cells. The percentage of apoptotic cells continued to increase with increasing

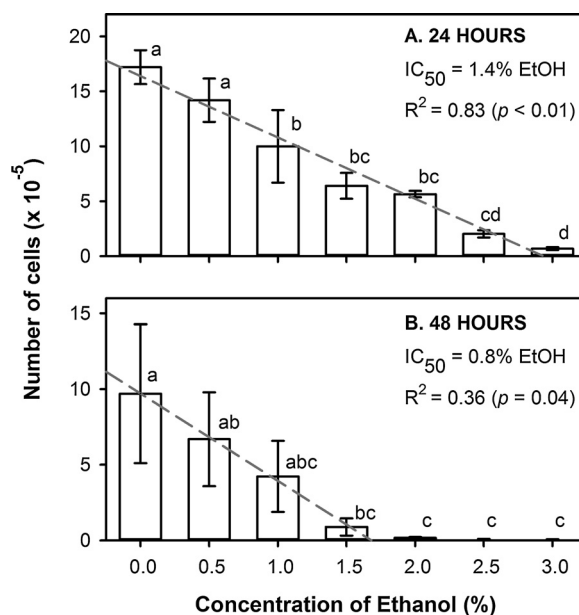


Fig. 1. Ethanol exposure reduces the number of viable undifferentiated mES D3 cells. Undifferentiated mES D3 cells were cultured with or without ethanol, as described in Section 2, and viable cell numbers were determined by light microscopy and trypan blue exclusion after 24 or 48 h (panels A or B, respectively). Each bar represents the mean number of viable cells over three independent experiments ($n = 3$) \pm SEM. Means with different letters are significantly different ($p \leq 0.05$). Dashed lines represent the regression of cell number by ethanol concentration within the ethanol range of 0–3% after 24 h (panel A) or 0–1.5% after 48 h (panel B). The values of R^2 and estimates of IC_{50} are also shown in each respective panel. The slope of each regression line was significantly different from zero ($p \leq 0.05$).

ethanol concentrations to approximately 2.5-fold over control levels (considering both early and late apoptotic cells) at 3% ethanol. An IC_{50} for cell viability after 8 h of 3.73% ethanol was estimated using the regression line [$y = (-12.5)(x) + (84.3)$] for the dose–response trend, the slope of which was significantly different from zero ($p = 0.004$). Similarly, after 24 h of culture (Fig. 2B), a significant 75% decrease of viable cells accompanied by a significant 4-fold increase of apoptotic cells was observed at 2% ethanol. Higher ethanol concentrations resulted in greater apoptotic responses. An IC_{50} for cell viability after 24 h of 2.61% ethanol was estimated using the regression line [$y = (-24.0)(x) + (93.0)$] for the dose–response trend, the slope of which was significantly different from zero ($p < 0.001$). After 48 h of culture (Fig. 2C), a significant 30% decrease of viable cells was observed at 1.5% ethanol, and further significant decreases were observed at higher ethanol concentrations. A significant increase of apoptotic cells was also observed, beginning at 2% ethanol, but, unlike at 8 and 24 h, the increase at 48 h was exclusively reflected in the late apoptotic fraction of cells. Higher ethanol concentrations resulted in greater apoptotic responses which peaked at 3% ethanol with approximately 95% of cells expressing either the early or late apoptotic phenotype. An IC_{50} for cell viability after 48 h of 1.99% ethanol was estimated using the regression line [$y = (-25.7)(x) + (84.3)$] for the dose–response trend, the slope of which was significantly different from zero ($p < 0.001$).

3.1.3. Ethanol exposure for 10 days is cytotoxic to undifferentiated mES cells

Prior to examining the effects of ethanol on mES cell differentiation, the cytotoxicity of ethanol in undifferentiated mES cells was examined over a period of 10 days, the same approximate time-frame used in differentiation studies. As shown in Fig. 3, relative to controls, no significant change in MTT reduction was observed at ethanol concentrations up to 0.5%. However, a significant increase of MTT reduction, depicted as an apparent decrease

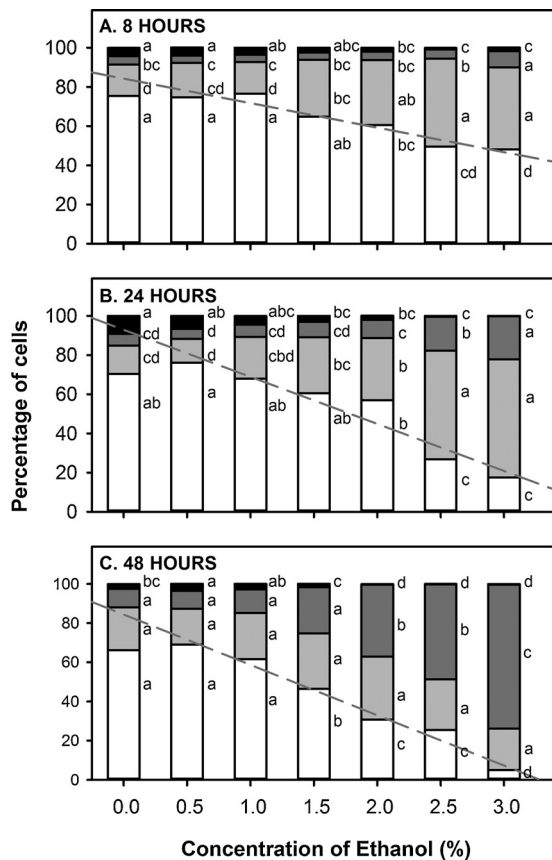


Fig. 2. Ethanol exposure induces apoptosis in undifferentiated mES D3 cells. Undifferentiated mES D3 cells were cultured with or without ethanol, as described in Section 2, and apoptotic cells were identified after 8, 24, or 48 h (panels A, B, or C, respectively) using flow cytometry. Each bar within a stack represents the mean percentage of unstained (white), early apoptotic (light gray, annexin V⁺ only), late apoptotic (dark gray, annexin V⁺/PI⁺), or necrotic (black, PI⁺ only) cells over three independent experiments ($n=3$). Means with different letters are significantly different ($p \leq 0.05$). Dashed lines represent the regression of unstained cell percentages by ethanol concentration within the ethanol range of 0.5–3% in each panel. The values of R^2 were 0.41, 0.75, and 0.79 for panels A, B, and C, respectively. The estimates of IC_{50} were 3.7%, 2.6%, and 2.0% ethanol for panels A, B, and C, respectively. The slope of each regression line was significantly different from zero ($p \leq 0.05$).

in cytotoxicity, was observed at 0.75% ethanol. This hormetic effect was not observed at higher ethanol concentrations. Rather, a significant decrease of MTT reduction, indicating apparent cytotoxicity, was observed beginning at 1.25% ethanol and reached a maximum of approximately 100% cytotoxicity at 1.5% ethanol. Because MTT reduction is a reflection of overall metabolic rate, for our purposes

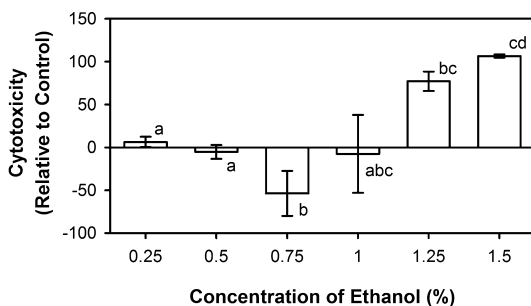


Fig. 3. Ethanol exposure alters MTT reduction by undifferentiated mES D3 cells. Undifferentiated mES D3 cells were cultured with or without ethanol, as described in Section 2, and cytotoxicity was measured after 10 days using an MTT-reduction assay. Each bar represents the mean percent cytotoxicity over two independent experiments ($n=10$) \pm SEM. Means with different letters are significantly different ($p \leq 0.05$).

we considered both increases and decreases in MTT reduction to be evidence of cytotoxicity. Therefore, in subsequent differentiation studies, ethanol concentrations no higher than 0.5% were used.

3.2. Ethanol inhibits differentiation of mES cells to cardiomyocytes

To assess the effects of ethanol exposure on mES cells during differentiation, undifferentiated mES cells were stimulated to form cardiomyocytes in the presence or absence of ethanol for 11 days as described in Section 2.6. In the absence of ethanol, this culture technique was sufficient to trigger differentiation of D3 mES cells as suggested by decreased expression of *Oct-3/4* and *Nanog* from day 0 to day 5 of culture by 2.3-fold and 3.4-fold, respectively.

Rhythmically contracting cells, indicating differentiation into cardiomyocytes, were observed on day 11 of culture (Fig. 4A and B). For D3 and J1 control cultures (not containing ethanol), approximately 37% and 76% contained contracting cells, respectively. The presence of ethanol during the 11 day culture period led to a dose-dependent decrease in wells containing contracting cells with significant 52% and 70% decreases observed in D3 cultures containing 0.4% and 0.5% ethanol, respectively, and significant 68% and 72% decreases observed in J1 cultures containing 0.3% and 0.5% ethanol, respectively. No significant change in the fraction of cultures containing contracting areas was observed at ethanol concentrations lower than 0.3% for either cell line.

The number of cells recovered per culture on day 11 was determined for both D3 and J1 cells, the fraction of cultures containing viable cells was determined for J1 cells, and the percentage of cells expressing the myosin heavy chain was determined for D3 cells. The presence of ethanol at concentrations of 0.3% or higher led to an apparent decrease of viable D3 cells per culture by 64% or more (Fig. 4B), but this decrease was not significant at any one ethanol concentration. When compared collectively, the number of viable D3 cells in cultures exposed to ethanol at 0.3% or higher was significantly lower than that for cultures exposed to less than 0.3% ethanol ($p=0.03$). For J1 cells, ethanol at concentrations of 0.3% or higher caused a significant decrease of cell number per culture by 30% or more (Fig. 4E). Concentrations of ethanol lower than 0.3% had no significant impact on J1 cell number per culture. These results suggest that ethanol at 0.3% or higher reduces the frequency of contracting cells, at least in part, by reducing the number of cells during differentiation. This possibility is supported by the observation that ethanol concentrations of 0.3% or higher caused a loss of all cells from 31% or more of the D3 cell cultures over the 11 day culture period (Fig. 4F). The presence of ethanol at concentrations of 0.3% or higher led to an apparent decrease of MHC expression by D3 cells (Fig. 4C), but this decrease was not significant at any one ethanol concentration. When compared collectively, the expression of MHC by D3 cells exposed to ethanol at 0.3% or higher was significantly lower than that for cells exposed to less than 0.3% ethanol ($p < 0.01$).

To assess the effects of ethanol exposure during the first two days of culture only on subsequent mES cell differentiation, undifferentiated J1 cells (without LIF) were stimulated to form cardiomyocytes as above but with the presence or absence of ethanol during the hanging drop stage only. Rhythmically contracting cells were observed on day 11 in 64% of control (no ethanol) cultures (Fig. 5A). The presence of ethanol at concentrations up to 0.5% had no significant affect on the fraction of contracting areas when assessed between individual treatment groups or when assessed collectively for groups treated with $<0.3\%$ or $\geq 0.3\%$ ethanol. Similarly, exposure to ethanol during the hanging drop phase at concentrations up to 0.5% had no significant affect on the number of viable cells per culture (Fig. 5B) or the fraction of surviving cultures (Fig. 5C).

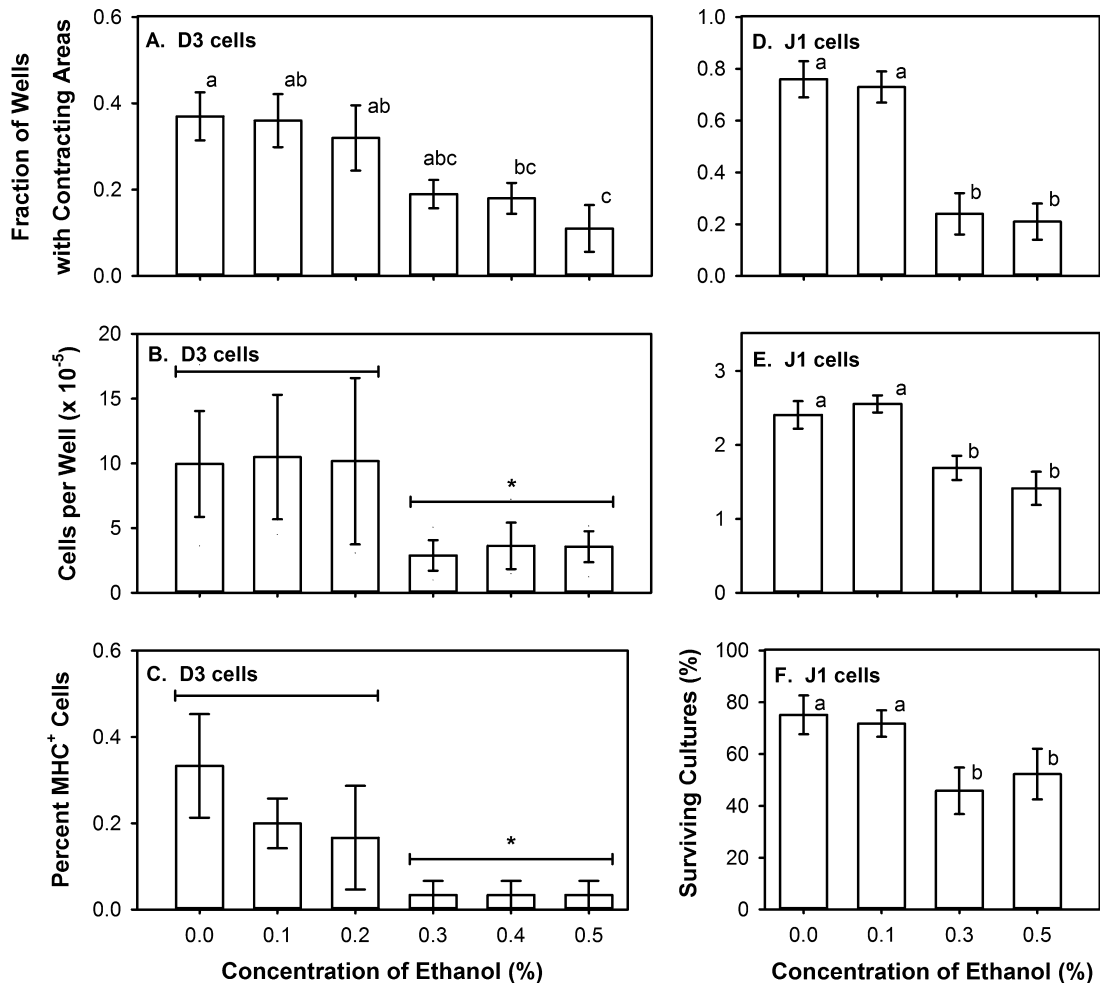


Fig. 4. Ethanol exposure inhibits mES cell development into cardiomyocytes. Undifferentiated mES D3 or mES J1 cells were cultured without LIF and with or without ethanol for 11 days using the hanging-drop method to stimulate development of cardiomyocytes. Each bar represents the mean (\pm SEM) over three (mES D3, $n=3$, Panels A–C) or nine (mES J1, $n=9$, Panels D–F) independent experiments. Panels A and D: the fraction of viable mES cell cultures (wells) within an experiment that contained contracting areas observable by light microscopy on day 11. Panels B and E: the number of viable cells per culture (well) on day 11. Panel C: the percent of mES D3 cells expressing MHC (MHC⁺) as measured using flow cytometry on day 11. Panel F: the fraction (%) of cultures established on day 0 that contained viable cells on day 11. Means with different letters are significantly different ($p \leq 0.05$). * Indicates the mean of all data within treatments of 0.3% ethanol or higher is significantly different from the mean of all data within groups of <0.3% ethanol.

4. Discussion

Ethanol exposure has been found to cause cell death in embryonic cells, human ES cells, and mES cells [9–11,19–21]. Many of these studies examined only one or a small number of ethanol concentrations that were not sufficient to estimate dose–response relationships. In the present study, a clear linear dose–response was observed for ethanol-induced loss of viable, undifferentiated mouse D3 ES cells after both 24 and 48 h (Fig. 1). The ethanol IC₅₀ values for viable cell numbers decreased from 24 to 48 h (1.4% and 0.8%, respectively) indicating a time-dependent, as well as a dose-dependent, sensitivity to ethanol toxicity in these cells. The bulk of the cell loss was likely attributable to apoptosis. A significant increase in early apoptotic cells was observed after eight hours of ethanol exposure (Fig. 2), and the percentage of apoptotic cells increased both in a time- and dose-dependent manner throughout the 48 h observation period. The estimated viable cell IC₅₀ values of 3.7%, 2.6% and 2.0% ethanol after exposures of 8, 24, and 48 h duration, respectively, were based on data within the ethanol range of 0.5–3%. Control cell data were excluded from these calculations because of the high background level of annexin V binding. That background was most likely an artifact of the protease/EDTA method used to detach and dissociate adherent ES cells prior to

staining and flow cytometric analysis, a phenomenon that varies in degree depending on the cell line used [22–31].

Ethanol-induced apoptosis in mES cells has also been reported by others. Huang et al. [10] exposed B5 mES cells (strain 129/Sv) to 0.63% (v/v) ethanol for 4 h and found a significantly increased incidence of apoptosis. By contrast, Arzumayan et al. [9] exposed undifferentiated E14Tg2A mES cells (strain 129/Ola) to a similar concentration of ethanol (approximately 0.58%) for 48 h but found no significant increase in apoptosis. An explanation for these disparate results could lie in the inherent sensitivity of the different mES cell lines to ethanol-induced apoptosis and/or the genetic background based on the mouse strain from which they were derived. The D3 mES cell line used in the present study was derived from the 129S2/SvPas strain of mouse, and these cells appear to have low sensitivity to ethanol under the culture conditions used, relative to the B5 and E14Tg2A lines, because significant increases in apoptosis were observed only at concentrations of 1.5% and higher. Alternatively, it is possible that variations in medium components between studies could underlie part of the differential sensitivity of these cells. For example, the amount of serum used in the present study (12.5%) was lower than that used by either Huang et al. [10] (20%) or Arzumayan et al. [9] (15%). Similarly, the reducing agent 2-mercaptoethanol (0.1 mM) was a component

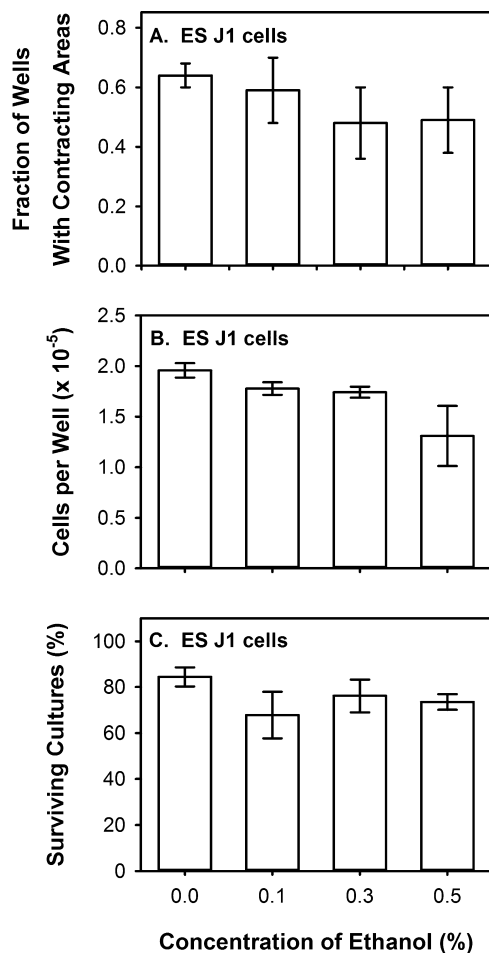


Fig. 5. Ethanol exposure during the hanging drop period of culture fails to inhibit development into cardiomyocytes. Undifferentiated mES J1 cells were cultured without LIF for 11 days using the hanging-drop method to stimulate development of cardiomyocytes. Ethanol was added to some cultures but only during the first two days when cells were suspended in hanging drops. Each bar represents the mean (\pm SEM) over seven independent experiments ($n=7$). Panel A: the fraction of viable mES cell cultures (wells) within an experiment that contained contracting areas observable by light microscopy on day 11. Panel B: the number of viable cells per culture (well) on day 11. Panel C: the fraction (%) of cultures established on day 0 that contained viable cells on day 11.

of the medium used by Arzumnyan et al. [9] as well as in the present study, whereas it was not present in the medium used by Huang et al. [10] who found the greatest sensitivity to ethanol. Withdrawal of 2-mercaptoethanol alone from mES cell cultures has been shown to increase oxidative stress and reduce the viability of those cells [32,33]. Moreover, ethanol exposure has been associated with increased production of reactive oxygen species, oxidative stress, and enhanced apoptosis in mES cells and other cells [10,21,34,35]. Therefore, the presence of 2-mercaptoethanol in culture medium may mask the toxic potency of ethanol, a concern that might be raised when testing any chemical capable of inducing oxidative stress.

In the present study, differentiation of D3 and J1 mES cells over an 11 day culture period with simultaneous exposure to ethanol at concentrations of 0.3% or higher led to significantly reduced numbers of cultures containing rhythmically contracting cells when compared with untreated control cells (Fig. 4A and D). This was likely due, at least in part, to inhibited differentiation into cardiomyocytes, as suggested by reduced expression of myosin heavy chain (Fig. 4C), and the dysregulation of transcription factors associated with lineage commitment (shown by others) [9,21,36,37]. This

affect was also likely the result of cell loss (Fig. 4B, D and E). The lack of cytotoxicity evident for undifferentiated mES cells exposed to 0.5% ethanol in the MTT assay (Fig. 3) contrasts with the loss of mES cells exposed to less than 0.5% ethanol during differentiation. These data support the suggestion that differentiating mES cells are more sensitive to ethanol toxicity than are their undifferentiated counterparts, an observation also reported by others [9]. This point is highlighted by the observation that a slightly higher ethanol concentration (0.75%) caused significantly greater MTT metabolism by undifferentiated mES cells, a potentially hormetic response. Enhanced MTT metabolism by undifferentiated cells may have been a consequence of enhanced cell proliferation, a response that was observed in undifferentiated human ES cells after a week of ethanol exposure, albeit at a much lower concentration (0.12%) [38].

To further explore the time-frame of ethanol sensitivity leading to inhibited cardiomyocyte formation, we exposed mES J1 cells to ethanol during the first two days of culture only (the hanging drop stage). When compared to control cultures, no significant change was observed in the frequency of rhythmically contracting cells or the number of cells per culture at ethanol concentrations up to 0.5% (Fig. 5). These results suggest that ethanol-induced changes in mES cell differentiation are mediated predominantly by mechanisms that are triggered later in the differentiation process. This is supported by studies that examined ethanol's impact on mES cell differentiation down the neuronal path [36,37,39]. Two studies that examined slightly longer time-frames for ethanol affects on mES cell differentiation measured the expression of pluripotency markers and apoptosis. Arzumnyan et al. [9] found a significant 3-fold increase in the percentage of apoptotic cells in the embryoid bodies of ethanol-exposed (0.58%) differentiating mES cells after three days of culture, but no similar increase in apoptosis was evident among ethanol-exposed undifferentiated cells. These results suggest that ethanol-induced loss of mES cell viability may be triggered beginning on day three of differentiation. Arzumnyan et al. [9] also found that 0.58% ethanol caused an attenuation of the normal decline in the expression of SSEA-1, Oct-3/4, Sox-2, and Nanog by differentiating E14Tg2A mES cells over the course of six days. Lower concentrations of ethanol were examined by Adler et al. [11] who reported an attenuation of the decline of Oct-4 expression by differentiating mES D3 cells exposed to ethanol at 0.25% with a significant 3-fold difference from control cultures being observed after seven days. These studies and others [36,37] suggest that continuous ethanol exposure past the embryoid body stage may inhibit further differentiation of mES cells.

The impact of in vitro ethanol exposure on embryonic and other stem cells from humans and other species has been examined, and many parallels with mouse ES cells have been observed. In undifferentiated human ES cells, ethanol exposure at 0.12% for 24 h was sufficient to enhance apoptosis, decrease Oct-3/4 expression, and increase SSEA-1 expression [21]. Other differentiation markers were shown to be dysregulated by ethanol in Rhesus monkey ES cells [40]. Indeed, altered expression of numerous genes and/or proteins as well as metabolites have been reported in human and mouse ES cells, neural stem cells, induced pluripotent stem cells, pluripotent carcinoma cells, or cells derived from those cells [36–50]. The concentrations of ethanol that were most commonly used in these studies were in the range of 0.1–0.5% with the justification that 0.12% is equivalent to DUI levels and 0.29–0.5% falls within the levels measured in alcoholics [51]. The results of some of these studies support the suggestion that ethanol's toxicity varies with the developmental state of the cells exposed. In human embryonic stem cells or carcinoma cells and the embryoid bodies derived from those cells, ethanol exposure (up to 0.29% for 48 h) altered protein expression and DNA methylation profiles

in both undifferentiated and differentiated cells, but the patterns differed between the two differentiation states [41,42]. Numerous other studies have found that when ethanol exposure has been applied for multiple days-weeks during the development of human ES cells, the differentiation program is altered, as it is for mouse ES cells [36–38,43–46].

The only specific developmental endpoints examined in the present study related to differentiation into cardiomyocytes. As such, no specific conclusions can be made with regard to the success of other developmental lineages that may be found within the mixed populations obtained. However, based on the significantly reduced total number of cells recovered after 11 days of ethanol exposure at 0.3% or higher concentrations, it is likely that the number of cells within other lineages may have also been reduced. Taken together, these data suggest that the use of ethanol at concentrations of 0.3% or higher in culture systems similar to the EST, which induce the formation of cardiomyocytes, may cause the loss of cells and/or inhibited differentiation independent of other test chemicals. Adler et al. [11] have recommended using ethanol at concentrations lower than 0.25% in the EST, and the data presented here support that recommendation.

Conflict of interest

The authors do not have conflicts of interest that could be perceived to bias the work described in this manuscript.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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