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Research Article

ELEVATED *IGF2* EXPRESSION AND ENHANCED MOUSE BLASTOCYST DEVELOPMENT FOLLOWING *IN VITRO* EXPOSURE TO ETHANOL

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Summary: Low concentrations of ethanol applied *in vitro* during mouse preimplantation are known to have a stimulating effect on blastocyst outgrowth, cavitation and implantation. In the present study, we optimized culturing conditions in P1m (P1 culture medium) for the development of mouse embryos from the C57BL/6 strain. The conditions for incubation were tested at different concentrations of EDTA (0-500 μM) and at different pH. The supplementation of 100 μM EDTA and a pH of 7.3 were found optimal for the incubation. In the optimized P1m, morulas and early blastocysts at 3.5 dpc were treated with ethanol for 4 hours at concentrations ranging from 0.01% to 1.0% (v/v). The highest stimulating effect was observed at a concentration of 0.2%. Real-time PCR was performed to explore changes in the expression of the *Igf2*/*H19* imprinted locus. Ethanol applied at a concentration of 0.2% up-regulated the *Igf2* promoter P0 expression and showed a tendency to increase the *Igf2* promoter P1 expression. We did not detect any expression of *H19* at this developmental stage. Our data reveal that ethanol can alter the expression of the imprinted growth-promoting *Igf2* gene after *in vitro* cultivation of mouse preimplantation blastocysts and this is probably one of the mechanisms underlying the acceleration of mouse embryonic development.

Keywords: Blastocyst, embryo, ethanol, *H19*, *Igf2*, mice.

Abbreviations: ATPase - adenylpyrophosphatase; Cq - quantification cycle; dpc - days post coitus; E- efficiency; PCR- polymerase chain reaction; P1m - P1 culture medium; REST- relative expression software tool.

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INTRODUCTION

Cell membranes are very permeable to ethanol, which facilitates its entry into the blood stream, followed by a spread in all tissues. If applied during the early stages of pregnancy, alcohol can easily pass into the lumen of the uterus (Fabro et al., 1984) as well as the oviducts (Sandor et al., 1981). Thus, the preimplantation

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embryos can be exposed to its influence.

The effect of ethanol on the embryonic development depends on doses, duration of exposure, stage of development, and the manner of application, i.e. *in vitro* or *in vivo*. The effect of ethanol on the developing embryo after *in vitro* treatment during the preimplantation stage has been extensively studied in the last decades. However, quite variable results have been obtained, showing either stimulation or inhibition of cell growth and differentiation. The observed effect largely depends on the dose applied in treatments. High ethanol doses (≥220mM or 1%) usually lead to an inhibition of development (Leach et al., 1993). In contrast, stimulation of growth and formation of blastocysts resulting from activation of trophoblast cells differentiation have been observed within certain ranges of lower ethanol concentrations (Stachecki et al., 1994; Armant and Saunders, 1996; Wiebold and Becker, 1987). In *in vitro* experiments, ethanol partially regains the growth rate of mouse embryos to levels close to *in vivo* development (Leach et al., 1993; Stachecki and Armant, 1996). Blastocysts derived from zygotes treated with 0.1% ethanol develop faster and contain more cells similar to blastocysts treated with growth factors (Schultz et al., 1993).

With regard to the possible mechanisms involved in the stimulatory effect of ethanol, the imprinted *Igf2/H19* locus is of particular interest. It is located on mouse chromosome 7 and at position 11p15.5 in humans and plays a crucial role in embryogenesis. *Igf2* is a paternally expressed gene encoding a mitogenic factor. It has a growth regulating activity and is very similar in structure to insulin, known as the "insulin-like growth factor II" (Tycko and Morison, 2002). *H19* is a maternally expressed transcription modulator. It is one of the most expressed genes during the embryogenesis and is critical for the embryonal development and physiology. Previously, we found down-regulation of *Igf2* expression in placentae after *in vivo* treatment of mouse embryos at 3.5dpc with different doses of ethanol (Taseva et al., 2015a) and up-regulation in the developing embryos (Taseva et al., 2015b) in the middle of pregnancy.

In the present study, we focused on the effect of different doses of ethanol applied *in vitro* with the aim to establish the most stimulating dose for preimplantation embryo development. Although the stimulatory effect of certain concentrations of ethanol has been subject to intensive research, there is no data concerning the relationship between ethanol-induced acceleration of embryogenesis and changes in the expression of the growth-related imprinted locus *Igf2/H19* following ethanol exposure during preimplantation. The culture conditions for growth of C57BL/6 mouse embryos in P1m were optimized by adding EDTA, a widely used chelator of metal ions, known to increase blastocyst development as a component of the *in vitro* medium (Abramczuk et al., 1977; Chatot et al., 1989; Gardner and Lane, 1996).

MATERIALS AND METHODS

Embryo collection and culture

Culture medium optimization

C57BL/6 females, 2-3 months old were superovulated by intraperitoneal injection of 8-10 IU of a follicle

stimulating hormone (Folligon, Intervet Ltd., Cambridge, UK) followed after 42-48 h by injection of 5-6 IU of human chorionic gonadotropin (Chorulon, Intervet Ltd., Cambridge, UK). After chorulon treatment, the mice were immediately paired for mating overnight with males from the same mouse strain. Animals were kept at 21°C and a 12h light/12h dark regime. After detection of vaginal copulatory plug (0.5dpc) embryos were obtained at the two-cell stage (which was a guarantee that they were fertilized) by flushing the oviduct in Dulbecco's phosphate buffer saline (Sigma). The embryos were cultured at 37°C for 96 h until reaching blastocyst stage in sealed tubes containing 500 µl P1m (Preimplantation Stage One Medium, Irvine Scientific) optimized by adding 400 mg/100ml albumin (Sigma) (Nagy et al., 2003) and different concentrations of EDTA (cell culture tested-Sigma, FW=416.20) within the range of 0-500 µl. P1m contained $NaHCO_3$ and needed CO_2 incubation. A filtered gas mixture of 5% O_2, 5% CO_2 and 90% N_2 (Penkov et al., 1995) was used to maintain optimal pH (7.2 - 7.4).

Exposure to ethanol

The same animals kept under the same conditions, but without the procedure of superovulation in females, were used for alcohol treatment. Embryos in the beginning of 3.5dpc were collected by flushing both the uterine horn and the oviduct in Dulbecco's phosphate buffer. Only embryos at the morula or early blastocyst stage were cultured at 37°C for 4 h in sealed tubes containing 500 µl P1m optimized by adding 400 mg/100ml albumin, 100 µl EDTA, ethyl alcohol (absolute ethanol for analysis 99.9%, Valerus, Bulgaria) at different concentrations (0.01%, 0.1%, 0.2%, 0.4% and 1.0%, v/v) and the gas mixture for optimal pH (7.2-7.4) mentioned above.

RNA extraction and reverse transcription

Quick-RNA Micro Prep kit (Zymo Research) for high-quality total RNA from a single to 10^6 cells was used (blastocyst contained 60 to 120 cells) as described by the manufacturer. Genomic DNA contamination was removed by including the optional step for DNase I treatment. The final RNA elution step was performed by adding 6 µl of RNase-free water preheated to 95°C.

cDNA was synthesized using Sensiscript Reverse Transcription (Qiagen) optimized for <50ng RNA. The eluted RNA solution from blastocysts was added to a reaction mixture containing 2 µl 10x buffer RT, 2 µl dNTP Mix (5 mM each dNTP), 2 µl Oligo dT (10 µM), 1 µl RNase inhibitor (10 U/µl), 1 µl Sensiscript Reverse Transcriptase and RNase-free water to a final reaction volume of 20 µl and incubated at 37°C for 60 min. Immediately after the reaction, the synthesized cDNA was frozen at -20°C.

Real-time PCR

Real-time PCR was performed on cDNAs using Maxima SYBR green qPCR master mix (2x) and PikoReal 96-Real-time PCR system (Thermo Fisher Scientific, USA) *Hsp90ab1* and *Atp5b* were used as an endogenous reference for normalization of the qPCR data. The PCR cycling program for all promoters of *Igf2* and *H19* included 10 min at 95°C,

followed by 40 cycles of 30 s at 95°C, 30 s at 58 or 59°C (annealing temperatures of the oligos are shown in Table 1), and 30 s at 72°C. Oligo sequences for *Hsp90ab1*, *Atp5b* and *Igf2*- P0, P1, P2, P3 were according to Taseva et al. (2015a) and for *H19* according to Kharroubi et al. (2001). Oligo sequences, product length and annealing temperatures are presented in Table 1.

The relative expression ratio was calculated based on E of the target gene and E of the endogenous reference calculated by using the equation $E = 10^{(-1/slope)}$ (Rasmussen, 2001). Deviations of Cq values (ΔCq) for the target and reference genes were obtained by subtracting averaged Cq values of the sample from averaged Cq values of the calibrator: (CqCalibrator) – (CqSample). The relative fold change was calculated by using the equation $((ETarget)^{\Delta Cq\ (Target)})$ / $((EEnd.\ ref.)^{\Delta Cq\ (End.\ ref.)})$ (Pfaffl, 2001).

Statistical analysis

Sample means from control and experimental groups after treatment with ethanol or EDTA were compared for significance of difference by using GraphPad Prism 5 with one-way analysis of variance (ANOVA) and Newman-Keuls and Dunnett's post-hoc tests. Statistical analysis of data from Real-time PCR was performed using the REST (relative expression software tool) (Pfaffl, 2002). Significant differences between the means (±SE) are indicated by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

RESULTS

Optimization of the *in vitro* culture medium

pH optimization

Our results showed that the optimal pH was approximately 7.3 (7.2-7.4), when P1m became light raspberry red in color. Almost 60% of the embryos developed normally from the single cell to eight-cell stage at pH varying within the range 6.9-7.5. Embryos remained insensitive to changes in the acidity of the medium up to the eight-cell stage, but thereafter, optimal acidity appeared critical for normal development. At pH<7.2 (6.9-7.2) the percentage of morulas and blastocysts sharply decreased, their development slowed down, they collapsed and finally died. At pH 7.5 embryonic development also slowed down, some embryos

Table 1. Primary sequence, product length and annealing temperature of the oligos used in Real-time PCR analysis.

GENE	FWD Primers (5'-3')	REV Primers (5'-3')	Ta/°C	Prod.
Igf2 P0	ATTGACCCAGCCAGCGGATC	CTGTACTCTAGTCGCTTCGTAG	59	125
Igf2 P1	CTCGTCACTTCTCCTACGGTG	CCCAGTCGTTTTCCTGGACAC	59	135
Igf2 P2	GTTCTGTCCCGTCGCACATTC	GGTATGCAAACCGAACAGCG	59	102
Igf2 P3	CTGGACATTAGCTTCTCCTGTG	CTGAAGTTGGGTAAGGAGGC	59	88
H19	CCACTACACTACCTGCCTCAGGAATCTGC	GGTGGGTACTGGGGCAGCATTG	58	522
Atp5b	GGCCAAGATGTCCTGCTGTT	GCTGGTAGCCTACAGCAGAAGG	59	207
Hsp90ab1	GCTGGCTGAGGACAAGGAGA	CGTCGGTTAGTGGAATCTTCATG	59	175

exhibited asynchronous and abnormal development, indicating that the alkaline character was even more toxic to the embryos. The changes in embryonic development occurring at suboptimal pH were irreversible. The embryos did not restore their development upon a shift to the optimal pH range.

EDTA Optimization

Further, we studied the effects of EDTA applied at different concentrations on the development of two-cell embryos to eight-cell, morula and blastocyst stage. The addition of EDTA at concentrations ranging between 5 and 200 μM did not affect significantly the development of two-cell to eight-cell embryos. A statistically significant developmental deviation compared with controls was observed only at the high dose of EDTA (500 μM) when the mean number of embryos decreased to 81%.

Embryo development from the eight-cell stage to morula in the presence of 5-200 μM EDTA was not significantly different compared with the control group. The dose of 100 μM EDTA was optimal for embryo development as 100% of embryos developed to the morula stage. Statistically significant differences between treated and untreated embryos were established only for the high EDTA concentration (500 μM).

All embryos (100%) developed from the morula stage to blastocysts after treatment with doses of 50 and 100 μM EDTA while there was a decrease in development to this stage at all other concentrations. At a concentration of 50 μM EDTA the percentage of embryonic development was approximately 97% (92-100%). The optimal EDTA concentration was 100 μM when 98% (95-100%) of the embryos developed to the blastocyst stage. The percentage of blastocysts decreased with increasing the EDTA concentration in the medium. At a concentration of 500 μM EDTA the percentage decreased to 68% (53-82%) and significantly differed from the controls as well as from all other groups (Table 2).

Table 2. Influence of different concentrations of EDTA within the range 0-500 μM on embryonic development from two-cell to blastocyst stage during preimplantation. Significant differences between the means (±SE) are indicated by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Volume medium [μL]	Volume EDTA [μL]	Molarity EDTA [μM]	Number of embryos [n]	2-cell stage [%]	8-cell stage [%]	Morula [%]	Blastocyst [%]
500.0	0.0	0	46	100	97	91	89
497.5	2.5	5	42	100	97	92	90
495.0	5.0	10	50	100	98	94	93
475.0	25.0	50	55	100	98	97	97*
450.0	50.0	100	47	100	98	98	98*
400.0	100.0	200	52	100	96	89	85
250.0	250.0	500	48	100	81*	74**	68***

Ethanol treatment

Effect of ethanol on the development of blastocysts

The analysis started at the beginning of 3.5dpc, with an average of 50% of the embryos reaching the early blastocyst stage (41.6-58%). No effect on growth and development was observed at ethanol concentrations lower or equal to 0.01%. However, there was a statistically significant rise in the percentage of formed blastocysts (by 16.7%) with the increase of ethanol concentration to 0.1%. The optimal ethanol concentration, which resulted in the highest stimulatory effect, was 0.2%, when the percentage of development to blastocysts reached 26%. At this concentration we observed large expanded blastocysts with transparent blastocoel reaching the phase of "hatching" (Fig. 1). A decrease in the number of morulas started in the presence of 0.5% ethanol, when blastocysts developed to a more advanced stage. The maximum ethanol dose of 1.0% lowered the percentage of developed blastocysts compared with the controls (about 4%), thus showing a toxic effect (Table 3). The differences between ethanol concentrations of 0.1% and 0.2% as well as 0.2% and 0.5% were found statistically significant by ANOVA.

Effect of ethanol on the Igf2/H19 expression

In this assay we used only expanded blastocysts at 3.5dpc treated with the optimal ethanol dose (0.2%) for 4 h. Our data showed that the expression of *Igf2*-P0 in ethanol-treated blastocysts increased 1.36-fold ($p<0.05$) compared with controls, whereas the *Igf2*-P1 expression increased 1.25-fold ($p<0.08$) (Fig. 2). The *Igf2*-P2 oligos annealed to the DNA template from blastocysts with low specificity amplifying nonspecific sequences, which could reduce the reliability of results. For that reason, these oligos were excluded from the assay. There were no significant differences in the *Igf2*-P3 expression levels. At this developmental stage, *H19* showed no expression in control or treated blastocysts.

DISCUSSION

Cultivation of embryos under *in vitro* conditions may adversely affect their growth and development, as they are outside the optimal conditions of the mother's organism. Culture medium composition, acidity, gas mixture and humidity are factors, which affect optimal

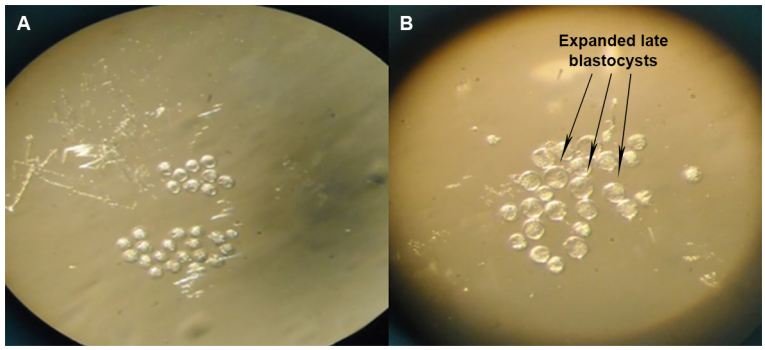

Figure 1. Blastocysts after 4 h of cultivation at 3.5dpc. Control blastocysts (A); blastocysts after treatment with an optimal dose of 0.2% ethanol (B).

Table 3. Effect of different concentrations of ethanol on blastocysts development after treatment for 4 h at 3.5dpc. Significant differences between the means (±SE) are indicated by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Medium volume [µL]	Ethanol volume [µL]	Percent ethanol in medium [%]	Number of embryos tested [n]	Percent of blastocyst before cultivation [%]	Percent of blastocyst after cultivation [%]	Difference before and after cultivation [%]
500.00	0.00	0.00	76	51.0	61.0	10.0
499.95	0.05	0.01	72	50.0	59.0	9.0
499.50	0.50	0.10	78	41.3	58.0	16.7*
499.00	1.00	0.20	65	50.0	76.0	26.0**
497.50	2.50	0.50	58	58.0	70.2	12.2
495.00	5.00	1.00	59	48.0	52.0	4.0*

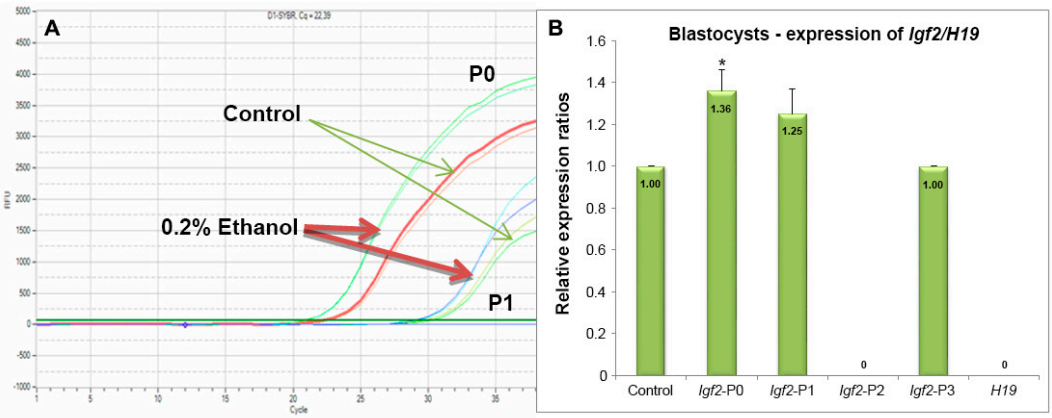

Figure 2. Graphical presentation of Real-time PCR amplification curves of *Igf2*-P0 and P1 promoters in untreated and treated blastocysts (A); mRNA expression levels of *Igf2* promoters and *H19* gene in blastocysts treated with 0.2% ethanol for 4 h (B).

embryogenesis. Since the development of the first Whitten's medium in the last century, media for *in vitro* embryo cultivation have been constantly optimised in order to match better with *in vivo* development. The requirements for the culturing media are increasing with the aim to not only ensure optimal development to implantation, but also normal development of vital embryos until their birth, without alterations in their genetics and epigenetics.

Improvements in the preimplantation development due to the presence of EDTA at optimal concentrations in the medium have been demonstrated in previous research (Gardner et al., 1997). In the present study, we analyzed the concentration range where EDTA can stimulate embryonic development (10-100 µM) at both eight-cell and morula stages. A statistically significant difference in the development from morula to blastocyst was observed at concentrations of 50 and 100 µM EDTA, compared with controls (Table 2). Our results correspond to results

from previous studies showing that EDTA concentrations between 10 and 100 μM are optimal (Mehta, 1990). Interestingly, one study showed that 10 μM EDTA had the most stimulating developmental effect (Abramczuk, 1977), while other authors observed the best effect at 100 μM EDTA (Mehta et al. 1993; Gardner and Lane, 1996; Matsukawa et al., 2002).The latter result was also confirmed in our study.

The specific effect EDTA has on the development of different mouse strains depends on the different components present in the culture medium. The synergistic effect of EDTA and the amino acids in the culture medium promote a high rate of blastocyst development (Gardner, 1996). A concentration of 100 μM EDTA in a medium with added amino acids yields a rate of embryo development, which is similar to the *in vivo* development (Mehta and Kiessling, 1990), while human serum reduces the effect. This could partly explain the tenfold difference in the optimal doses applied in different studies.

The acidity of the culture medium is another important prerequisite for proper *in vitro* development, critical to the cultivation process. It is affected by the amino acid composition, since different amino acids contribute to the overall pH with their different acidity. In most commercial media the optimal pH range is between 7.2 and 7.4, which is consistent with our results.

In the present study, we investigated the impact of different doses of ethanol on the development of mouse blastocysts *in vitro* (Table 3). Our results confirmed previously published data, which revealed a stimulating effect of ethanol in the range of 0.1-0.4% (Leach et al, 1993). The maximum effect was reached at a concentration of 0.2% ethanol. During the 4 h treatment, we observed large, expanded blastocysts developing into a "hatching" phase (Fig. 1), which was not detected for any other dose tested as well as for the controls. Such blastocysts are typical for 4.5dpc *in vivo* when the actual implantation takes place. In the present study, the comparative analysis between the tested concentrations showed significant differences between 0.1% and 0.2% ethanol indicating a dose dependent effect on development. The latter concentration has not been tested previously, however, it shows an even higher potency for growth stimulation than the already described optimal concentration of 0.1% ethanol. Significant differences were found also between treatments with 0.2% and 0.5% ethanol, suggesting that the optimal stimulating action of ethanol can be observed within narrow limits.

The threshold concentration for alcoholic toxicity in mammals is 0.1% ethanol (Abel, 1984). This threshold was based on early studies that showed reduced growth and variation in differentiation during organogenesis in embryos treated with ethanol at concentrations over 0.2-0.3% (Brown et al., 1979; Priscott et al., 1982). Our data showed that the toxicity threshold was higher since the inhibitory effect occurred at concentrations of 0.4% to 1.0%. A complete inhibition of preimplantation development has been observed by others at concentrations significantly above this threshold, whereas four-cell embryos cultivated in a medium with 1.6% ethanol were still capable of forming blastocysts (Leach, 1993). The inhibitory effect of high doses of ethanol may be due to increased membrane fluidity,

inhibition of K-Na ATPase, membrane-associated enzymes or altered membrane receptor system properties (Sun and Sun, 1985). High doses of ethanol are cytotoxic and alcohol can physically destroy cell membranes (Littleton et al., 1989).

In vitro embryonic development is slower in comparison to *in vivo* development, possibly due to the lack of optimal conditions and embryogenesis-regulating factors produced by the mother's organism. *In vitro* application of ethanol partially accelerates the rate of growth to levels close to *in vivo* conditions (Leach et al., 1993; Stachecki and Armant, 1996). Ethanol-stimulated embryos have increased rates of cell division and cell migration compared with controls, and more rapidly form blastocysts, which are released from "zona pellucida".

There are various hypotheses for the accelerating *in vitro* effect of ethanol during the preimplantation stage. According to some authors, this process may result from changes in the intracellular signaling through biochemical pathways regulating the rate of embryonic growth. Ethanol acts by releasing and increasing levels of intracellular Ca^{2+}, which is one of the major mediator molecules in signal transduction pathways regulating cell growth and differentiation (Florman et al., 1992; Kline and Kline, 1992). It was reported that Ca^{2+} levels in the morula increased immediately after the addition of 0.1% ethanol in the medium (Stachecki and Armant, 1994). Increased trophoblast differentiation is also associated with increased mitotic rate. It was also reported that levels of c-myc, an important transcription factor, increased fourfold in blastocysts after 5 min exposure to 0.1% ethanol (Leach et al., 1994).

In the present study, following treatment with the optimal ethanol dose of 0.2% at 3.5dpc for 4 h, we observed a change in *Igf2* expression in parallel with the strong stimulatory effect. There was a statistically significant increase in *Igf2*-P0 expression as well as a non-significant trend for increase of *Igf2*-P1 expression (Fig. 2B). In our experiments, expression of *H19* was not detected in either the treated embryos or in the controls (Fig. 2B). This is consistent with data from previous studies where *H19* was either very poorly expressed or not expressed at the blastocyst stage (Haycock, 2007). An increase in *Igf2* expression levels has also been reported after treatment of embryonic stem cells with varying concentrations of ethanol (Villanueva, 2013). Similarly, a dose-dependent rise in *Igf2* was observed in *in vitro* human trophoblast cell cultures exposed for 72 h to ethanol at concentrations in the range 5-90 mg/dl (Clave et al., 2014). The authors also showed that at low ethanol doses the significant increase in expression was after 72 h, whereas at high doses such an effect was reported after 1 h of cultivation.

It is interesting to note that ethyl alcohol, among all types of alcohols, is the only one that can accelerate preimplantation embryogenesis. Structurally similar alcohols such as methanol, propanol, isopropanol or butanol do not exhibit such an effect. All of these alcohols inhibit embryonic development in a dose-dependent manner at concentrations between 0.05% and 1.0% (Kowalczyk et al., 1996).

In conclusion, the preimplantation development of mouse C57Bl/6 embryos can be optimized by addition of EDTA in P1m at a concentration

of 100 μM and by maintaining pH at approximately 7.3. The addition of ethanol at various concentrations to the medium may accelerate or inhibit the embryonic development depending on the dose applied. The stimulating effect of ethanol was observed within a narrow concentration range. It was highest at 0.2%, outperforming the previously published dose of 0.1%. To our knowledge, we report for the first time that ethanol added at optimal stimulating concentrations *in vitro* at the preimplantation stage can alter the expression of the growth-related imprinted *Igf2* gene. This is probably one of the mechanisms underlying the higher rate of cell division and the ethanol-mediated acceleration of embryonic growth in this phase of pregnancy.

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