Can Ethanol Enhance the Cleavage and Development of Blocked Two-cell Embryos?

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Abstract—**Arresting in a certain step of development, like two-cell stage could be one of the reasons of infertility. In this study, we evaluate the effects of ethanol on growth and development of mouse two-cell arrested embryos. In this xperimental study 4-6 week-old female mice were coupled with males following superovulation by intraperitoneal injection of pregnant mere serum gonadotropin (PMSG). Positive vaginal plug mice were killed 48 hours after human chorionic gonadotropin (hCG) injection. Two-cell embryos were transferred to culture medium, and divided in three groups, 1(control 1), 2 (control 2) and 3 (experimental). Second and third groups were exposed to 4 ℃ for 24 hours in order to arrest the two-cell embryos. Group 2 were incubated immediately in 38 ℃, while group 3 were exposed to 0.1% ethanol for five minutes and group 1 were incubated without any exposure to low temperature. The developmental rate of embryos exposed to low temperature (4 ℃) were significantly decreased and retarded (P=0.001). There was no significant difference in the mean percent of cleavage rate between groups, but the mean percent of degenerated embryos (P=0.045), morula formation (P=0.005), blastocyst formation (P=0.014) and hatched blastocyst (P=0.001) in 120 h study, were significantly different between groups. The effect of 0.1% ethanol on arrested two-cell embryos can significantly enhance the mean percent of morula formation and development of blastocysts and hatching blastocysts comparing to 2nd control group, without any significant effect on cleavage rate.**

Index Terms—**Block, ethanol, mouse, two-cell embryo**

I. Introduction

Infertility is one the most important problems among couples allover in the world, causes many social and psychological effects on couples [1]. However there are a wide variety of treatment options for couples experiencing infertility, from fertility drugs to assistive reproductive technologies. No activation of ovum by sperm [2], [3], reduction of growth rate in embryos and arresting in one stage of development like two-cell stage are some of infertility reasons in some couples [4]. The causes of infertility can involve either one or both partners. In about 20 percent of cases, infertility is due to a cause involving only the male partner. In about 30 to 40 percent of cases, infertility is due to causes involving both the male and female. In the remaining 40 to 50 percent of cases, infertility is entirely due to a cause involving the female [1].

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Activators can significantly increase cleavage and developmental rates for parthenogenetic embryos cultured in medium supplemented with some kinds of activators. Arresting in two-cell stage is one of the topics that are concerned by many researchers. In fact arresting of embryo at two-cell stage occure in some couples referring for ART to fertility and infertility centre probably because at the mid-two-cell stage definitive transcription from the zygotic genome occurs in the early embryo [5], [6].

The studies on fifty five different strains of mice have shown that there are significant differences for two-cell block in different strains. Among the factors contributing to this phenomenon, maternal factors play an important role [7], [8]. However the mechanism underlying arrest in two-cell stage is poorly understood, the authurs demonstrated that this developmental arrest is maternally controlled [9]-[11]

However, the sperm mitochondria, the microtubule-organizing center [MTOC] precursors and the stored cellular components of the sperm play no major role in cleavage-stage of embryogenesis. Thus, the early embryo is almost entirely dependent on the egg for its initial complement of the subcellular organelles and macromolecules that are required for survival prior to the robust activation of the embryonic genome at early cleavage-stage development. These maternal components are encoded by maternal-effect genes [12], [13].

Embryo arrest causes strong and effective changes in protein synthesis of embryo. During this process, maternal signals that cause the cleavage are blocked and embryos degenerate. Studies show that oocytes exposing to the media containing activators, resulted in a significant enhancement in the rate of cleavage and development [14]. A lot of genes were identified which are necessary for passing the embryonic developmental stages. The injection of ooplasm from one normal oocyte directly to the two-cell arrested embryo removes the blocking [15].

Last studies have indicated that ethanol can activate oocytes and causes parthenogenesis [16]-[18]. Ethanol with changing signaling pathway, controls the rate of embryogenesis and can affect the development of preimplantation stage embryos [19].

Furthermore it was shown that the ethanol-mediated increase in intracellular Ca2+ is immediate and that a period of exposure of 5 min is as effective as exposure for 24 h in stimulating development [16], perhaps because ethanol initiates a signal-transduction cascade that has an impact on subsequent development. The ability of ethanol to accelerate development at dissimilar stages from the one-cell to the blastocyst stage suggests that ethanol may affect key developmental regulatory mechanisms. It therefore appears

that there is a threshold concentration of ethanol for increasing intracellular calcium [16].

When oocyte exposes to ethanol, an intracellular rise in Ca2+ is attained by an increased permeability of cell membrane to Ca2+ and enhancement of intracellular Ca2+ activates the oocyte. Ethanol with contribution to function as secondary messengers, such as calcium, can stimulate the embryos development before implantation. Arresting in two-cell stage is one of the topics that are concerned by many researchers. It has shown that the most of inbred and outbred embryos of different strains of mice were arrested in two-cell stage of development. The studies on fifty five different strains of mice have shown that there are significant differences in two-cell stage arresting in different strains [15], [16]. Among the causes of this case, maternal factors are more important than paternal factors [20].

However, the sperm mitochondria, the microtubule-organizing center [MTOC] precursors and the stored cellular components of the sperm play no major role in cleavage-stage embryogenesis. Thus, the early embryo is almost entirely dependent on the egg for its initial complement of the subcellular organelles and macromolecules that are required for survival prior to the robust activation of the embryonic genome at cleavage-stage development. These maternal components are encoded by maternal-effect genes [12], [13].

Also, the studies show that the rate of early cleavage depends on reserved mRNA and proteins of oocytes. Arresting causes strong and effective changes in protein synthesis of embryo. During this process, maternal signals that cause the cleavage are blocked and development arrests and embryos degenerate. Studies show that oocytes exposing to the media containing activators, resulted in a significant enhancement in the rate of cleavage and development [14].

Under laboratory conditions, because of environmental stresses, cell death [apoptosis] increases [21].

Keeping embryos in low temperatures causes DNA fragmentation and cell death [22], [23]. Last studies have indicated that ethanol can activate oocyte and causes parthenogenesis. Scientists have attempted to suppress the arresting in two-cell stage by transferring embryos to uterus [24] and adding the chelators containing heavy metals [e.g. EDTA] and cytoplasm of F1 hybrid embryos into the medium of arrested two-cell embryos. There are some methods for activation of ovules successfully [25], [26]. These methods are divided to physical methods, such as electrical activation and chemicals, such as ethanol [27], [28], methanol [29], [30], calcium ionophore [31] and strontium [32]. Some of the growth factors contribute in cellular signaling passway and regulate growth and differentiation, so adding these factors to the medium could stimulate the development.Ethanol with changing signaling passway, controls the rate of embryogenesis and can affect the development of preimplantation stage embryos [16], [33].

Other ovule activators are benzyl alcohol, propanediol and methanol. In electrical activation very expensive machineries are necessary, hence, there is a requirement for a cheaper and easier method. Developmental arrest can be considered as one of the reason for infertility. Ethanol is a classic teratogen that is capable of inducing a wide range of developmental abnormalities that vary in severity. This research was aimed at how ethanol can activate the arrested two-cell mouse embryos. The aim of this study was to evaluate and comparison of the effects of ethanol and strontium on growth and developmental rate of two-cell arrested mouse embryos exposed to low temperature.

II. Materials and Methods

In this experimental study, 6 to 8 weeks of age mice (NMRI strain, N=150) were prepared from Razi Vaccine & Serum Research Institute and in order to adapt with the new environment, they were kept in the animal house of Arak University of Medical Sciences under standard conditions (12 h light, 12h dark, 21 ± 5 ℃) for one week. In order to stimulate the superovulation, female mice were intraperitoneally injected with 10 i.u. PMSG (Pregnant Mare Serum Gonadotropin) (Folligon,Intervet,U.K.) followed 48 hours latter by 10 i.u. hCG (Human Chorionic Gonadotropin) (Intervet, U.K.). The females were then coupled with males, and with detection of vaginal plug (indication of successful mating) they were separated and killed by cervical dislocation method (according to ethical committee).

Next, their uterine tubes were transferred to RPMI medium. Approximately 40h post hCG two-cell embryos were collected in RPMI medium by flushing uterine tubes and after three times washing transferred to 25 microliter drops of M16 medium (Sigma, M-7292) that covered with liquid paraffin. Subsequently 2-cell embryos were divided into three groups, 1st (control 1), 2nd (control 2) and 3rd (experimental) groups. The embryos of 1st control group just incubated in 5% Co2 in air at 37℃ for 120 hours without any exposing to low temperatures. The 2nd Control and 3rd experiment groups were exposed to 4℃ for 24 hours and kept in refrigerator in order to induce arresting in two-cell embryos. Then, 2nd Control group were incubated immediately while 3rd group were exposed to medium containing 0.1% ethanol for 5 minutes [16] and were incubated for 120h after three times washing in M16 medium.

A. Embryo evaluation

Embryos were observed periodically at 200x on an inverted microscope and graded for stage of development including degenerated embryos, 3-4 and 5-8 cell embryos, morula, blastocyst, and hatching at 48h, 72h, and 96h post cooling. Half of the culture medium was replaced with fresh medium at 24h intervals. Embryos that didn,t progress to the next cleavage stage were seperated at the time of each medium chang.

B. Statistical analysis

The proportions of embryos reaching to 3-8 cell, morula, and blastocyst stages were recorded. Each reported data point represents an observation involving at last 3 replicates. All statistical analyses of this study were analyzed by SPSS 11.5 software. Growth rate and developmental parameters of embryos were analyzed by one-way ANOVA. Significant data was evaluated by Post Hoc and differences between groups were determined.

III. Results

The embryos of groups 2 and 3, which were exposed to 4 ℃ for 24 hours, reached to blastocyst stage in 18 to 24 hours later than 1st control group. Growth and development of embryos are shown in table 1. There was no significant difference between the mean percent of cleavage rate among groups (P=0.844), but the mean percent of degenerated embryos was significantly different among groups (P=0.045) over a 72 hours assay. Also, the mean percent of degenerated embryos were significantly different between groups 1 and 2 (P= 0.037) but never shown between groups 1 and 3 or 2 and 3. One-way ANOVA shows that the mean percent of morula formation over a 72 h assay was significantly different between groups (P=0.005), but never shown between groups 1 and 3 or 2 and 3. Data analysis after 120 h assay shows that the mean percent of blastocysts were 77.7±5.9, 61.06±10.9 and 72.3±2.7 in groups 1, 2 and 3 respectively and statistically, there were significant differences between groups (P=0.014) but never shown between groups 2 and 3 lonely. The mean percent of hatched blastocyst (P=0.001) during 120 hours assay were significantly different between all groups even 2 and 3 lonely. The developmental rate of embryos exposed to low temperatures(4 ℃) were significantly arrested (P=0.001).

TABLE I: The Mean Percent of Degenerated Embryos, Cleavage, Morula, Blastocysts And Hatched Blastocysts in Groups 1, 2 and 3

Time	**72h**			**120h**		**Total no.of embryos**
Groups	Degenerated embryos	Cleavage	Morula	Blastocysts	Hatched blastocysts	
Group 1	9±5.9 [n=9]	31.7±13.4 [n=29]	59.3±16.5 [n=54]	77.7±5.9 [n=71]	60.2±6.5 [n=55]	238
Group 2	34±14 [n=51]	34.6±13.9 [n=48]	31.3±8.7 [n=46]	61±10.9 [n=88]	9±13.4 [n=19]	252
Group 3	22.2±8 [n=12]	37.6±14.8 [n=23]	40.2±12 [n=24]	72.3±2.7 [n=53]	51.4±16.4 [n=29]	141
	P=0.045	P=0.844	P=0.005	P=0.014	P=0.001	

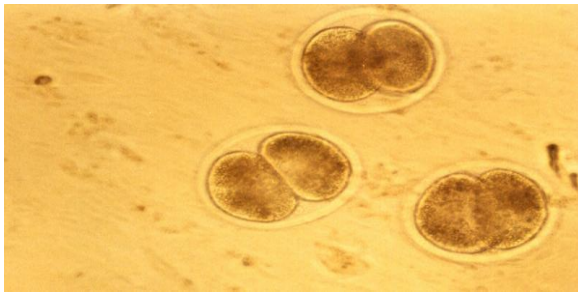

Fig. 1. Two-cell embryos after flushing are shown by inverted microscope [×400 magnificent]

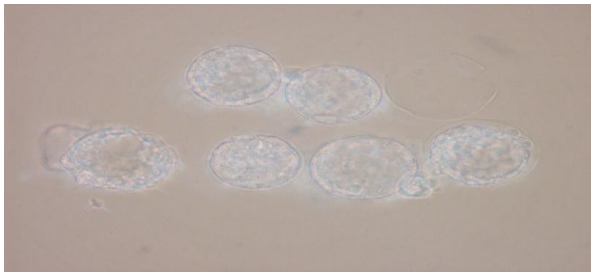

Fig. 2. Blastocyst and hatched blastocyst stages after 120 hour are shown by inverted microscope [×400 magnificent]

Two-cell embryos have shown in figure 1 after flushing of uterine tube and the blastocyst and hatched blastocyst stage embryos in figure 2 after 120 hours.

IV. Discussion

In this study a model of preimplantation embryo development in vitro showed that a brief exposure to 0.1% ethanol enhances the developmental progression of cultured mouse two-cell blocked embryos. These data support previous observations of accelerated preimplantation embryonic development after exposure to ethanol at earlier stages in vitro [16]. This study shows that arrested two-cell embryos expose to 0.1% ethanol can significantly enhance the mean percent of morula formation and development of blastocysts and hatching blastocysts stage comparing to control group, without any significant effect on cleavage rate.

Ca2+ signaling at fertilization is a vital event for switching the oocytes from meiosis to mitosis [29], [34]. To date many artificial activation methods including ethanol have been developed and most of them cause a monotonic increase in intracellular Ca2+ [18], [33], [35], [36].

Ethanol stimulates mitosis, improves in vitro culture condition from fertilization through to blastocyst stage, enhances blastocel formation rates and increases the rate of hatched blastocyst from the zona pellucida. These developmental effects seem to be due, in part, to an observed transient increase in intracellular Ca2+ that can be replicated using A23187 [16]. These finding confirm our data resulted in the present study by a similar mechanism.

Manipulating Ca2+ signaling patterns during egg activation can severely alter mammalian preimplantation development and implantation outcome [37]. In addition it has been shown that the Ca2+ signal controls chromosome disjunction in early sea orchin embryos [38]. The intra cellular Ca2+ ossilation play a role not only in the resumption of meiosis but also in the subsequent embryo development [39].

The growth and developmental rate of in vitro produced embryos was slower and lower than in vivo because of lacking maternal growth factors and related nutrients in vitro. These materials regulate the process of embryogenesis [40].

In a research, some of two-cell mouse embryos were exposed to 4 ℃, showing an increasing in developmental failure and degenerated embryos, and they reached to blastocyst stage 18 to 24 hours later than control group that confirm our results [12]. It seems in 4 ℃ temperature, cellular metabolism and protein synthesis of embryos decreased and metabolic transport system was affected. Probably under this

condition, after a short time embryotrophic factors change to embryotoxic factors and increase the sensitivity of embryos to unfavorable compounds of culture. Probably with low temperature exposure of embryos, the membrane permeability maybe altered and cause acumulation of some embrotrophic factors in one side of the membrane and cause a toxic effect. Interestingly, the presence of amino acids in the culture medium was also responsible for an inhibition of embryo development, as amino acids spontaneously break down to produce embryotoxic ammonium ions [41], [42].

The growth and development of in vitro produced embryos was slower and lower than in vivo because some maternal growth factors and related nutrients in vitro are absent and sensitivity of developing early embryo to changes in their immediate environment effects on viability and development [43].

Our findings emphasizes on the fact that two-cell mouse embryos exposed to 4 ℃ undergoes developmental arrest and the rate of degeneration is increased and the time of blastocyst formation is retarded compared to 1st control group however the differences weren,t significant and their data havn,t shown. In contrast, decreasing of degenerated embryos for 3rd group in compare with 2nd group is probably because of activating effect of ethanol.

It has been demonstrated that metabolic activity of an embryos could be inhibited by cooling at refrigerated temperature for a short term storage meanwhile in a viable state. Experiments show that mouse fertilized oocytes can be stored at 48C for even 48 h cause temporary cessation of DNA synthesis with maintenance of potentiality to develop to normal blastocysts [44], [45].

The enhancement of degenerated embryos of group 2 in comparison with grup 1 is probably because of low temperature exposure (4 ℃). In contrast, decreasing of degenerated embryos of group 3 in comparison with group 1 is probably because of activating effect of ethanol. Rogers used 7% ethanol for 7 minutes in order to activate the parthenogenesis in embryos in preimplantation stage and the rate of cleavage, morula and blastocyst formation was 83, 79 and 72% respectively. In our study, these rates were 37, 40 and 72%. The reason for a decrease in cleavage and morula formation is related to degeneration of some embryos in response to exposing to low temperatures. The similar result about blastocyst formation, in addition to different concentration of ethanol and exposure time is probably because of more sensitivity and vulnerability of developmental stages to higher concentrations and longer exposure of ethanol as a teratogen [46].

Leach et al [47] showed that one or two-cell embryos cultivated in a medium containing 1.6% ethanol had some problems for reaching to blastocyst stage. Ethanol exposure either arrested or enhanced normal development, depending on dose and embryonic stage of exposure. Exposure of 1-cell and 2-cell embryos to 1.6% ethanol decreased blastocyst formation and hatching, and exposure of 1-cell embryos to 0.4% ethanol inhibited their development. At 0.1%, ethanol had an opposite effect, causing an increase in the percent of blastocyst formation of treated 1-cell and 2-cell embryos. Neither inhibition nor stimulation of blastocyst formation occurred in 4-cell embryos exposed to 0.1–1.6% ethanol. Blastocysts previously exposed to 0.1% ethanol during the 1-cell stage appeared to form adhesive trophoblasts earlier than control embryos, indicating that ethanol exposure can induce precocious differentiation of the trophoblast cells. The ethanol treated blastocysts contained significantly more cells than control blastocysts [47]. These results indicate that ethanol can alter preimplantation development by both inhibiting or accelerating cell growth and differentiation [47].

In another study, 0.1 to 0.8% of ethanol had no effect on blastocyst formation that is similar to our results [48]. Wiebold et al showed that 0.1% ethanol increases the rate of blastocyst formation to 86% in mouse two-cell embryos which has significant difference with our result due to low temperature exposure in the present study and decreased metabolic activity [44].

There were no effects of 0.1% ethanol on the percentage of embryos reaching the morula or expanding, hatching or hatched blastocyst stages at various times as compared to control embryos. Fewer embryos developed to these stages when cultured in 1.0% ethanol. Embryos cultured in 1.0% alcohol before transferring had lower implantation rates and lower fetal survival [49]. It is shown that chronic 10% ethanol intake by young female mice reduces the ovulatory response and impairs the quality of the oocytes. These findings show that chronic moderate ethanol ingestion by young female mice results in decreased fertilization, embryo growth retardation, cleavage arrest, and abnormal embryo development in vitro [50].

Grabiec et al. [51] showed that combination of magnetic field with ethanol has a commplementory effect on oocyte activation, cleavage and it,s parthenogenetic development.

Clearly, further study is required to identify the specific mechanism of the action of ethanol on embryonic cells and to determine whether exposure to ethanol can improve preimplantation development in other species. Possibly in the next studies in order to activation of arrested two-cell embryos, combination of other modality or protocol with ethanol have a better results for cleavage an development. At the end of this study we hope that the effects of other activators on two-cell embryos development are studied and compared in the future.

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