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The Effects of Ethanol on Growth and Development of Mouse Two-cell Arrested 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 experimental study 4-6 week-old female mice were coupled with males following superovulation by intraperitoneal injection of PMSG. Positive vaginal plug mice were killed 48 hours after HCG injection. Two-cell embryos were transferred to RPMI medium; cultured in M16 medium and divided in three groups: 1(control 1), 2 (control 2) and 3(experimental). Second and third groups were exposed to 4ºC for 24 hours in order to arrest the two-cell embryos. Group2 were incubated immediately in 38ºC, while group3 were exposed to 0.1% ethanol for five minutes and group1 were incubated without any exposure to low temperature. The developmental rate of embryos exposed to low temperature (4ºC) 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 control group, without any significant effect on cleavage rate.**

Keywords-Infertility; Ethanol; Mouse; Two-cell Embryo; Blastocyst

I. INTRODUCTION

Infertility is one the most important problems among couples in the whole world, causes many social and psychometrical effects on couples [1]. There are a wide variety of treatment options for couples experiencing infertility today, from fertility drugs to assistive reproductive technologies. In some countries as well as Iran, many studies are being performed in order to identify the causes and treatment ways of infertility. 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. No activation of ovule 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]. 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 [5, 6]. The studies on fifty five different strains of mice have shown that there are significant differences in two-cell stage arresting in different strains. Among the causes of this case, maternal factors are more important than fraternal factors. 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 stops and embryos die. Exposing embryos to low temperatures is one of the causes of development arresting. The effect of low temperature (4 ºC) on implantation has been studied and indicated that cell division is arrested in two-cell embryos and after transferring to the optimum conditions, they show lower rates of development. Under laboratory conditions, because of environmental stresses, cell death (apoptosis) increases [7]. Keeping embryos in low temperatures causes DNA fragmentation and cell death [8, 9]. Scientists present a new method named "vitrification" that is much better than cryopreservation method. Vitrification is ultra-rapid IVF embryo freezing instead of the traditional slow freezing process. Vitrification in IVF can allow freezing of spare embryos with better post-thaw survival rates and higher pregnancy and live birth rates from frozen embryo transfer cycles [10]. Last studies have indicated that ethanol can activate ovule and causes parthenogenesis. Scientists have attempted to suppress the arresting in two-cell stage by transferring embryos to uterus [11] 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 [12, 13]. These methods are divided to physical methods, such as electrical activation and chemicals, such as ethanol [14, 15], methanol [16, 17], calcium ionophore [18] and strontium [19]. 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 effect the development of preimplantation stage embryos [20, 21]. Ethanol with concentration higher than 5% activates ovules and causes parthenogenesis. When ovule exposes to ethanol, the permeability of cell membrane increases to calcium and enhancement of intracellular calcium activates the ovule. Ethanol with contribution to function of secondary messengers, such as calcium, can stimulate the embryos development before implantation. 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. 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.

II. Materials And Methods

In this experimental study, 6 to 8 week-old 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 room of Arak University of Medical Sciences under standard conditions (12 h light, 12h dark, 21 ± 5 ºC) for one week. In order to stimulate the superovulation, 10 units of PMSG (Pregnant Mare Serum Gonadotropin) were intraperitoneally injected to females. Then, females were coupled with males, and positive vaginal plug mice were separated and killed (cervical dislocation method) 48 hours after HCG (Human Chorionic Gonadotropin) intraperitoneal injection. Next, their uterine tubes were transferred to PRMI medium. Two cell embryos were collected in RPMI medium by flushing uterine tubes and after three times washing in RPMI medium, transferred to 25 microliter drops of M16 medium (Sigma, M-7292) that covered with liquid paraffin and divided into three groups:1 (control 1), 2 (control 2) and 3 (experimental). The embryos of control 1 group just incubated in 38ºC for 120 hours without any exposing to low temperatures. Control 2 and experiment groups were exposed to 4ºC for 24 hours and kept in refrigerator in order to induce arresting in two-cell embryos. Then, Control 2 group were incubated immediately while experiment group were exposed to medium containing 0.1% ethanol for five minutes. 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 4ºC for 24 hours, reached to blastocyst stage in 18 to 24 hours later than group 1. The results of 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. Analysis of results after 120 h 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 were significantly different between all groups even 2 and 3 lonely. The developmental rate of embryos exposed to low temperatures (4 ºC) were significantly arrested (P=0.001). The effects 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 stage comparing to control group, without any significant effect on cleavage rate. Fig. 1 shows the two-cell embryos after flushing and Fig. 2 shows the blastocyst and hatched blastocyst stages after 120 hours.

IV. Discussion

The growth rate of embryos in vitro condition was slower and lower than in vivo conditions because of lacking maternal growth factors and related nutrients. These materials regulate the process of embryogenesis. In a research, some of two-cell mouse embryos were exposed to 4ºC, showing an increasing in developmental arresting and degenerated embryos, and they reached to blastocyst stage 18 to 24 hours later than control group that confirm our results. It seems in 4ºC temperature, cellular metabolism and protein synthesis of embryos decreased and metabolic transport system was affected. Probably under this condition, embryotrophic factors change to embryotoxic factors after a short time and increase the sensitivity of embryos to unfavorable compounds of culture. Also, lacking in essential components heightened with passing time. In this stage, with changing the components of medium and adding some growth factors and regulation of buffering system, we increase the time of preservation and keep them safe and alive [22]. The enhancement of degenerated embryos of group 2 in comparison with group 1 is probably because of exposing to low temperatures (4ºC). 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 [23]. Leach 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. These results indicate that ethanol can alter preimplantation development by both inhibiting or accelerating cell growth and differentiation [24]. In another study, 0.1 to 0.8% of ethanol had no effect on blastocyst formation that is similar to our results [25]. Wiebold showed that 0.1% ethanol increases the rate of reaching to blastocyst stage to 86% in mouse two-cell embryos. 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 [26]. Our different results are due to low temperature effects on development. It is shown that chronic 10% ethanol intake by young female mice reduces the ovulatory response and impairs the quality of the oocytes. The 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 [27]. At the end of this study we hope that the effects of other activators on two-cell embryos development are studied and compared in near future.

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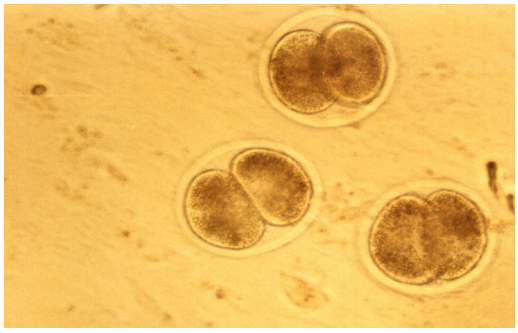

Figure 1. Two-cell embryos after flushing are shown by inverted microscope (×400 magnificent)

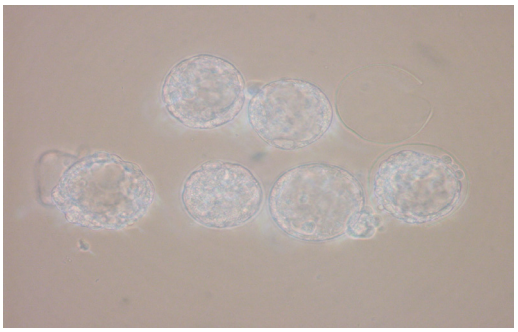

Figure 2. Blastocyst and hatched blastocyst stages after 120 hour are shown by inverted microscope (×400 magnificent)

TABLE I. THE MEAN PERCENT OF DEGENERATED EMBRYOS, CLEAVAGE, MORULA, BLASTOCYSTS AND HATCHED BLASTOCYSTS IN GROUPS 1, 2 AND 3.

Time	**72 h**			**120 h**		**Total of embryos**
Groups	**Degenerated embryos**	**Cleavage**	**Morula**	**Blastocysts**	**Hatched blastocysts**	
Group1	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
Group2	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
Group3	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	