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ORIGINAL ARTICLE

Comparison of Ethanol and Ionomycine for cleavage and development of blocked mouse two-cell embryo

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ABSTRACT

Introduction: One of the most important reasons of infertility between couples is arresting of embryos in certain stage of development like two-cell stage embryos. There are different methods for activating oocytes and embryos routinely used in fertility and infertility centers. In this study, the effects of ethanol and calcium ionophore on activating of arrested two-cell stage embryos were compared.

Methods: After superovulation, female and male mice were coupled and the positive vaginal plaque mice were killed. Their uterine tubes were washed in order to gather their two-cell embryos in M16 medium. Subsequently the two-cell embryos were divided randomly to four groups. The 1st (1st control) group were incubated without any exposure, and the other embryos were exposed to 4 °C for 24 h in order to arrest in two-cell stage. The 2nd group were incubated after low temperature exposure. The 3rd group were exposed to 0.1% ethanol for 5 minutes and the 4th group to 10 µM calcium ionophore for 5 minutes and subsequently were incubated.

Results: The mean percent of degenerated embryos was increased significantly with low temperature exposure between groups (P=0.005). Similarly the cleavage rate was significant between groups except between groups 3 and 4 (P=0.217). In addition, the developmental rate was significant between groups. The mean percent of hatched blastocyst were higher in group 3 compared to other groups but their differences were not significant (P=0.512).

Conclusion: Due to ethanol and calcium ionophore exposure of arrested two-cell embryos the rate of cleavage and blastocyst formation rate were increased probably because of calcium ossilation produced by chemical activators. Comparatively ethanol is more effectively on cleavage and development than calcium ionophore. *JOURNAL OF IRANIAN CLINICAL RESEARCH* 2015;1(2):*49-53*

INTRODUCTION

Infertility is the most important crisis of life that results in incidence of psychological problems and serious stress experiences for sterile patients. Because of the importance of infertility in some communities, many efforts have been done for its solution. In two recent decades major progresses in relation to diagnosis and treatment of infertility achieved and 65% of infertile couples succeeded to have a child (or children) by routine methods [1, 2].

Including the effective factors of sterility is inactivation of oocyte by sperm [3, 4] and decreasing the growth and development of embryos and arresting in a definite stage like two cell stage embryo [5]. This problem is one of the issues for challenging researchers. Inconvenient compositions in some mediums may be the reason of blockage [6].

To date, many challenges have been done for minimizing the potential extrinsic factors of embryo growth. Between the extrinsic factors, medium composition and oxidative metabolites are significant. Some of the intrinsic factors that cause arrest are genes, cytoplasmic factors and chromosomal renewal [7]. Embryonic arrest may be one of the mechanisms that inhibit development of the abnormal chromosomal and genomic inactivated embryos [8].

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Cleavage rate of embryonic cells depend on deposited protein and mRNA levels in oocytes. In two-cell stage, the internal maternal signals of cleavage are arrested due to sudden change in embryonic protein synthesis [9]. Among the effective factors of embryonic delay and arrest are exposing to low temperatures and chemical compounds like Hypoxanthin, Inosin, Adenosin etc. [10]. It was indicated that one of the embryonic arresting factors is low temperature exposure by 4°C. In temperature of 41 °C, developmental rate is significantly decreased [11]. In low temperature, exposure apoptosis of the embryonic cells will occur [12]. Low temperature exposure is one of the most important factors for embryonic lesions and DNA fragmentation [13].

There are physical, chemical and biologic methods for oocyte activation. In order to arrest in two-cell stage one of the biologic methods is completing the medium with F1 hybrid embryonic cytoplasm. In order to chemical methods, heavy metal chelator like EDTA [14, 15] and ethanol [16, 17], methanol [18, 19], ionophore and strontium [20-22] are used. Electroactivation is a physical method that has been used for oocyte activation. Exposing of two-cell embryo to calcium ionophore could increase the creb1 gene and thereby raising the embryo developmental rate [23].

In our previous study, the effect of ethanol on embryonic growth and development was assessed. Therefore, the aim of this study was the comparison of ethanol and calcium ionophore for growth and development of arrested embryos.

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 ºC) for one week [24, 25].

Animal experiments were approved by the Ethics Committee of Arak University of Medical Sciences and performed in accordance with University guidelines.

Ovulation induction

Superovulation has been done with i.p. injection of 10 iu PMSG (Intervet Holland) to female mouse. Then female and male mice were coupled and in the next morning positive vaginal plaque mouse were isolated and kept in another cage [26].

Positive vaginal plaque mouse sacrificed by cervical dislocation 48 hours after HCG injection and their oviducts transferred to RPMI 1640 (Gibco) medium. Subsequently two cell embryos were collected in RPMI by flushing of uterine tube, transferred to 25 µl droplets of M16 medium (Sigma, M-7292) under paraffin oil after three time washing. Then embryos divide to four groups. The 1st group (control group) incubated directly in 37 °C for 120 hours without any exposure. Another group was exposed primarily to 4°C (refrigerator) for 24 hours. The second group incubated after removing from refrigerator. The third group was exposed to medium containing 0.1% ethanol for 5 minutes and the fourth group to 10 µM calcium ionophore (Sigma C-7522) for 5 minutes [27, 28].

Evaluation of the embryos

The embryos were studied by stereo microscopy 72 and 120 hours after culture. The data were analyzed with one-way ANOVA using SPSS software (Chicago, IL, USA).

RESULTS

The two-cell arrested embryos were exposed to 4 °C for 24 hours had a 18 to 24 hours latency of blastocyst formation (the data were not shown).

The degenerated embryos were increased significantly by low temperature exposure in compare with 1st group but this was not significant between 3rd and 4th groups (P= 0.217).

Our evaluation during 72 hours showed that the mean percent of morula stage embryos was decreased significantly by low temperature exposure in compare with 1st group but was not significant between 3rd and 4th groups (P= 0.548) (Table 1).

The evaluation of blastocyst during 120 hours showed that there was significant difference between groups ($P \leq 0.05$). The rate of hatched blastocyt was lower significantly in groups 2, 3 and 4 in compare with 1st group but this value was not significant between 2nd , 3rd and 4th groups.

The cleavage rate in low temperature exposure groups (2, 3 and 4) was significantly lower than 1st group. Similarly the cleavage rate between 3rd and 4th group was significantly (P=0217) different. Each treatment repeated 3 to 5 times.

Table 1- Mean percent of degenerated, cleavaged and morula embryos in 72 h evaluation, blastocyst and hatched blastocyst of different groups in 120 h evaluation

	72 hour evaluation			120 hour evaluation		total
	Degenerated	**cleavage**	**morula**	**Blastocyst**	**hatched**	
Gr.1	3.6(15)[a] ± 11.85	88.14±3.60(111)[a]	3.7(94)[a]± 74.24	6.03(78)[a] ±61.42	8.41(58)[a] ±43.95	128
Gr.2	7.5(87)[d]±44.63	7.54(109)[c]±55.36	6.97(59)[c]± 30.80	[c])26(3.21±13.74	2.46(24)[c] ±12.21	196
Gr.3	3.63(32)[b,d] ±21.98	3.63(81)[d,d]±78.01	4.29(62) [c] ±58.76	[c])32(2.28±35.78	3.65(14) ±16.50	113
Gr.4	3.31(37)[d,b] ±28.43	3.31(133)[d,d]±71.56	4.36(100) [c]± 54.45	2.16(61)[b] ±28.69	1.96(29) ±12.66	170

Statistical analyses were down for developmental stage in each group: a is significant to b ($P<0.05$); a, b, d are significant to c ($P<0.001$); e is significant to f ($P<0.01$) and to g ($P<00.1$). C ($P<0.01$); d ($P<0.001$);

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DISCUSSION

This study showed that low temperature exposure of two-cell embryos resulted in arrest and increased degeneration and subsequently decreased cleavage and developmental rate. With effects of chemical activators on arrested two-cell embryos, the rate of degeneration decreased because cleavage and developmental rate were increased. Our data showed that with effects of ethanol in 3rd group, the cleavage rate is more than 4th group, similarly the rate of blastocyst formation was more than 4th group.

The cleavage rate of mouse two-cell embryos exposed to 4°C was decreased. Similarly the development was delayed, so that the authors reach to blastocyst 18 – 24 h latter than control group [29]. This finding is in concordance with our finding.

Two-cell blocked embryos can begin their growth and development by injection of ooplasm from high quality oocytes [30]. In fact, extrinsic and intrinsic embryonic factors may effect on embryo growth, development, and cause embryonic arrest. Culture media composition and oxidative metabolites are examples of extrinsic factors. Cytoplasmic factors, genes and chromosomes rearrangements are include intrinsic factors, which potentially cause embryonic arrest [31].

Keefer et al. reported that treatment of bovine oocyte with A23187 could improve developmental rate by microinjection method. Although they used microinjection but the results were similar with present study [32-34]. No toxic effect was observed in cleavage and development by short-time exposure with calcium ionophore, which is in concordance with present study [35].

Ethanol accelerates mitosis, enhance blastocyst formation, improves cavitation rates and raise hatched blastocyst from the zona pellucida. These developmental effects seem to be due, in part, to an observed transient increase in [Ca2+] that can be replicated using calcium ionophore [36]. Because of low temperature exposure, the rate of development in present study was lower than previous study [36]. This finding can describe the reason of high fleeting of embryos from morula to blastocyst and hatched blastocyst stage (in 3rd group).

The developmental progression of mouse two-cell embryos to the blastocyst stage was increased after culture in 0.1% ethanol for 3 days compared with control group (86% and 70% respectively) [37]. These data were different in comparison with present study because of low temperature exposure of embryos in present study.

Probably because ethanol commences 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 cause key developmental regulatory mechanisms [38].

High concentrations of ethanol (7%) could activate parthenogenesis in oocytes by inducing a single transient increase in [Ca2+]¡ [18, 19]

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possibly activation of blocked two-cell embryos in the present study is carried out by similar mechanism.

Rogers and et al., used ethanol for parthenogenetic activation of preimplantion embryos. They found that egg activation by means of ethanol was sufficient to produce high numbers of two-cell embryos. The effect on activation was clearly related to events after the first cell cycle because they found that approximately 70% of the two-cell embryos reached the blastocyst stage by ethanol activation. Their results were different with our results because of parthenogenetic activation of oocytes and their different applied concentration of activators [39].

The present study showed that embryos in 2nd group due to low temperature exposure, have low rate of growth and development compare with other groups. In this way, the mean percent of degenerated embryos in 2nd group is highest and blastocyst and hatched blastocyst is the lowest compare with other groups.

The present study showed that increasing rate of degenerated embryos in 3rd group compare with 1st group is due to 4 °C exposure and probably toxic effect of ethanol exposure in this stage of development although the duration exposure was very short.

Conclusion: Our data showed that ethanol (3rd group) could enhance the cleavage and development of arrested two-cell blocked embryos more effectively than 2nd and 4th groups but their difference in cleavage and hatched blastocyst were no significant compare with 4th group.

It is suggested that in the future study the quality of embryos produced by chemical activation be compare with control groups. Additionally the chromosomal abnormality of produced embryo by this method should study in the future studies because of their clinical application.

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