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ORIGINAL ARTICLE

Comparison of Ethanol and Ionomycine for Cleavage and Development of Arrested Two-cell Embryos

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ABSTRACT

Background: One of the most important causes of infertility is embryo arrest in a certain stage of development such as two-cell stage. Fertility centers use various methods for activating oocytes and embryos. In this study, we aimed to evaluate the effects of ethanol and calcium ionophore on activating embryos arrested at the 2-cell stage.

Materials and Methods: In this experimental study, after superovulation, female and male mice were coupled; then, those with positive vaginal plaque were sacrificed. Uterine tubes were rinsed in order to gather their two-cell embryos in M16 medium. Subsequently, the two-cell embryos were randomly divided into four groups. The first (1st control) group was incubated without any exposure. The second group was first exposed to 4°C for 24 h to arrest at two-cell stage, and then incubated after low temperature exposure. The third group was exposed to 0.1% ethanol for 5 min, and the fourth group was exposed to 10 µM calcium ionophore for 5 min and subsequently incubated.

Results: Due to ethanol and calcium ionophore exposure, the rates of cleavage and blastocyst formation increased in the arrested two-cell embryos probably because of calcium oscillation produced by chemical activators.

Conclusion: Ethanol was more effective in cleavage and development than calcium ionophore.

Key Words: Calcium ionophore, Ethanol, Infertility, Two-cell embryo

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INTRODUCTION

Infertility is one of the most important life crises that can result in psychological problems and serious stressful experiences for sterile patients. Given the importance of infertility in some communities, great efforts have been made for its solution. During the last two decades, major advancements have been made in relation to diagnosis and treatment of infertility and 65% of infertile couples succeeded to have a child (or children) via routine methods [1, 2].

One of the important causes of sterility is inactivation of oocyte by sperm [3, 4] and embryo arrest in a definite stage like two-cell stage [5], which poses a challenge for researchers. Inappropriate compositions in some media may be the reason for blockage [6].

To date, efforts have been made for curtail the potential extrinsic factors hindering embryonic growth. Among the extrinsic factors, medium composition and oxidative metabolites are significant. Some of the intrinsic factors causing 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].

Cleavage rate of embryonic cells is contingent upon deposited proteins 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]. Exposure to low temperatures and chemical compounds such as hypoxanthin, inosin, and adenosin can play a role in embryonic delay and arrest [10]. It was indicated that one of the embryonic arresting factors is low temperature exposure by 4°C. At 41°C, developmental rate is

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significantly decreased [11]. At low temperatures, exposure apoptosis of the embryonic cells occurs [12]. Low temperature exposure is one of the most important factors for embryonic lesions and DNA fragmentation [13].

There are physical, chemical, and biological methods for oocyte activation. To arrest at the two-cell stage, one of the biological methods is completing the medium with F1 hybrid embryonic cytoplasm. For this purpose, chemical methods and heavy metal chelators including EDTA [14, 15], ethanol [16, 17], methanol [18, 19], ionophore, and strontium [20-22] are used. Electroactivation is a physical method that has been applied for oocyte activation. Exposing two-cell embryos to calcium ionophore could increase the creb1 gene and thereby raise the rate of embryonic development [23].

The aim of this study was to compare ethanol and calcium ionophore in terms of 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 acquired from Razi Vaccine and Serum Research Institute. To adapt them to the new environment, they were kept in the animal house of Arak University of Medical Sciences under standard conditions (12 h light/dark, 21±5°C) for one week [24, 25].

Ovulation induction

Superovulation was performed using i.p. injection of 10 IU pregnant mare serum gonadotropin (PMSG; Intervet, the Netherlands) to female mice. Then, female and male mice were coupled and in the next morning positive vaginal plaque mice were isolated and kept in another cage [26].

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

Evaluation of the embryos

The embryos were studied by stereo microscopy 72 and 120 h after culture.

Statistical analysis

The data were analyzed using One-way ANOVA in SPSS, version 16. In all statistical measurements, P-value less than 0.05 was considered significant.

Ethical consideration

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

RESULTS

The two-cell arrested embryos were exposed to 4°C for 24 h and had a 18 to 24 h latency of blastocyst formation (the data were not shown). The degenerated embryos were increased significantly by low temperature exposure as compared with the first group, but the difference was not significant between the third and fourth groups (P=0.217).

Our evaluation at 72 h showed that the mean percentage of morula-stage embryos was decreased significantly by low temperature exposure as compared to the first group, but the difference between the third and fourth groups was not significant (P=0.548; Table 1).

The evaluation of blastocyst at 120 h showed a significant difference between the groups

Table 1. Mean percent of degenerated, cleavage, and morula embryos in 72 h evaluation, as well as 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	3.21 (26)[c] ±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 performed 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), g (P<00.1), c (P<0.01), and d (P<0.001).

(P≤0.05). The rate of hatched blastocysts was significantly lower in groups 2, 3, and 4 as compared to the first group, but the difference between the second, third, and fourth groups was not significant.

The cleavage rate in low temperature exposure groups (2, 3, and 4) was significantly lower than the first group. Similarly, the cleavage rate between the third and fourth groups was significantly different (P=0217). Each treatment repeated three to five times.

DISCUSSION

This study revealed that low temperature exposure of two-cell embryos resulted in arrest and increased degeneration, and subsequently, decreased cleavage and development rates. With effects of chemical activators on arrested two-cell embryos, the rate of degeneration diminished because cleavage and developmental rates increased. Our data showed that the cleavage and blastocyst formation rates were higher in the third group compared to the fourth group.

In a study by Bakhtiari et al., the cleavage rate of two-cell embryos exposed to 4°C was decreased. Similarly, the development was delayed, such that the they reached to blastocyst 18–24 h later than the control group [11]. This finding is in agreement with our results.

Two-cell blocked embryos can begin their growth and development by injection of ooplasm from high-quality oocytes [29]. In fact, extrinsic and intrinsic embryonic factors may affect embryonic growth and development and cause embryonic arrest. Culture medium composition and oxidative metabolites are examples of extrinsic factors. Cytoplasmic factors, genes, and chromosomal rearrangements are the intrinsic factors, which potentially cause embryonic arrest [30].

Keefer et al. reported that treatment of bovine oocyte with A23187 could improve rate of development through microinjection method. Although they used microinjection, their results were similar to ours [31-33]. No toxic effect was observed in cleavage and development by short-time exposure to calcium ionophore, which is in accordance with the present study [34].

Ethanol accelerates mitosis, enhances blastocyst formation, improves cavitation rate, and raises hatched blastocyst from the zona pellucida. These developmental effects seem to be due, in part, to an observed transient increase in Ca^{2+} that can be replicated using calcium ionophore [35]. Because of low temperature exposure, the rate of development in the present study was lower than the previous ones [35]. This finding can describe high fleeting of embryos from morula to blastocyst and hatched blastocyst stage (in the third group).

In a study by Webold and Beckers the developmental progression of two-cell embryos to the blastocyst stage was increased after culture in 0.1% ethanol for three days compared to the control group (86% and 70%, respectively) [36]. These findings were different from those of the present study because of low temperature exposure of embryos in the present study.

Ethanol's ability to accelerate development at dissimilar stages from the one-cell to the blastocyst stages suggests that ethanol may cause key developmental regulatory mechanisms [37].

High concentrations of ethanol (7%) could activate parthenogenesis in oocytes by inducing a single transient increase in Ca^{2+} [18, 19], possibly activation of blocked two-cell embryos in the present study occurred by a similar mechanism.

Rogers et al. used ethanol for parthenogenetic activation of preimplantion embryos. They found that oocyte 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 one-cell cycle because they found that approximately 70% of the two-cell embryos reached the blastocyst stage by ethanol activation. Their results were different from our results because of parthenogenetic activation of oocytes and the different applied concentrations of activators [38].

The present study showed that embryos in the second group had lower rate of growth and development compared to the other groups due to low temperature exposure. In the second group, the mean percentage of degenerated embryos was the highest and blastocyst and hatched blastocyst rates were the lowest in comparison with other groups.

The present study showed that increased rate of degenerated embryos in the third group compared to the first group is due to 4°C exposure and probably toxic effect of ethanol exposure in this stage of development although the duration of exposure was very short.

Our data revealed that the intervention in the third group could enhance the cleavage and development of arrested two-cell embryos more effectively than the second and fourth groups, but their differences in cleavage and hatched blastocyst were not significant compared to the 4th group.

Future studies are recommended to evaluate

the quality of embryos produced by chemical activation. Additionally, the chromosomal abnormality of the produced embryos by this method should be investigated in the future studies because of their clinical application.

CONCLUSION

Ethanol was more effective in cleavage and development than calcium ionophore.

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CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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