Effect of β-Mercaptoethanol with and without BSO (DL-Buthionine Sulfoximine) on Resumption of Meiosis, in vitro Maturation and Embryo Development of Immature Mouse Oocytes

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Abstract

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Introduction: The purpose of this study was to evaluated the effect of β-mercaptoethanol on resumption of meiosis, in vitro maturation of immature mouse oocytes and resulting embryo development with and without BSO (DL-Buthionine sulfoximine).

Material and Methods: Germinal vasicle (GV) were recovered from 6-8 weeks old NMRI ovaries and cultured in maturation medium in MEMα supplemented with 7.5IU/ml hCG, 100mIU/ml rhFSH, 5% FCS (control group) and adding 100μm β-mercaptoethanol (group 1) or with 5mM BSO + 100μm β-mercaptoethanol (group 2) for 24h. The matured oocytes then were fertilized and cultured for 5 days. Fertilization and development were accomplished in T6 medium.

Results: The percentage of GV oocytes reaching to metaphase I (or undergo GVBD) were 78.5%, 85%, 86% in control group, group 1 and group 2 respectively, that no significant difference was detected between groups. The proportion of oocytes that progressed to the metaphase II (MII) stage was minimum within 5mM BSO group (group 2) and maximum within β-mercaptoetanol group (group 1) with significant difference comparing with control and each other (P≤0.05). The percentage of embryos reaching to morula stage within β-mercaptoetanol group was significantly higher than the control group (5% and 12.2% respectively). None of oocytes treated with BSO could pass the 8 cell stage.

Conclusion: β-mercaptoetanol enhances IVM and improves embryo development. While adding BSO into the maturation medium even with β-mercaptoetanol decreases maturation and declines the embryo development.

Keywords: IVM, Mouse, Oocyte, BSO, β-mercaptoethanol

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Introduction

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS), and cellular antioxidant defense Generation of ROS occurs physiologically during cell metabolism. Under physiological conditions, oxidative phosphorylation in mitochondria is the main intracellular source of ROS, 2% of consumed oxygen (O_2) being incompletely reduced and generation $O_2^{\cdot-}$. Oxidative stress has been implicated in many different types of injuries, including membrane lipids peroxidation, oxidation of amino acids and nucleic acids, apoptosis and necrosis (1).

ROS is a term which encompasses all highly reactive, oxygen – containing molecules, including free radicals. There are several types of ROS including: superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot-}$) and singlet oxygen, which all are capable of reacting with membrane lipids, nucleic acids, proteins, enzymes and other small molecules, resulting in cellular damage (2). Higher quantities of $O_2^{\cdot-}$ and H_2O_2 have been detected in early cleavage stage mouse

embryos produced in vitro as compared to their in vivo counterparts (3-5) and have been related to early developmental arrest (5).
It is well known that oxygen tension within the lumen of the female reproductive tract is about one-third of what can be found under standard in vitro conditions (6).
The culture of embryos with a high oxygen tension in vitro (20%) may produce more free radical than embryo culture under 5% O_2 or 7% O_2(3,7). It appears that the balance between ROS production and scavenging is an important factor for the acquisition of fertilizing ability in vitro (8).
It has been reported that ROS induce mitochondrial dysfunction, DNA, RNA and protein damage (9) as well as inhibiting sperm – oocyte fusion (10). To protect oocytes and embryos from oxidative stress during in vitro culture, various antioxidants can be added to culture media (11) including enzymatic (e.g. superoxide dismutase, catalase and peroxidase) or non-enzymatic (e.g. GSH, vitamins C and E, scavengres) (12). Numerous studies have reported that exogenous administration of these enzymes, although acting only in the extracellular phase, could prevent some of the deleterious effects of ROS during embryonic development (5,15-17). mRNA transcripts for most enzymatic antioxidants have been detected in mammalian ooctyes and embryos (13,14).
Glutathione (GSH) is the major non protein sulphydryl compound in mammalian cells, it serves as a reservoir for cysteine and plays an important role in protecting the cell from oxidative damage (18). Synthesis of GSH during oocyte maturation has been reported in the mouse (19) hamster (20) pig (21) and cow (22) Glutathion content is increased during oocyte maturation in the ovary as the oocyte approaches the time of ovulation (24), thus, the levels of GSH found in oocytes at the end of maturation are considered as a good biochemical marker for oocyte viability (23).
Elevated levels of GSH has been reported to have beneficial effects on the promotion of the oocyte-spermatozoan complex to develop a MPN after IVF (25). Yamauchi and Nagai (1999) further demonstracted that a lack of GSH in oocytes prior to fertilization results in the inability to form a MPN after fertilization (26).
Law molecular weight thiols, such as cystemine and β-mercaptoethanol, could increase intracellular GSH synthesis when added during bovine IVM (27,28). Higher intracellular GSH content in oocytes could improve embryo-development and quality, and increase the number of embryos reaching the blastocyst stage on day 6 (29).
The influence of intracellular GSH synthesis on embryo development was supported by the finding that buthionine sulfoximide (BSO), a specific inhibitor of GSH synthesis, neutralized the primitive effect of thiol compounds on development and GSH synthesis of bovine oocytes and embryos (9,10). The objective of this work was to study the effect of β-mercaptoethanol with BSO on resumption of meiosis, in vitro maturation and embryo development.

Material and Methods

Reagents

All reagents were purchased from Sigma (Germany) except Gonal-F (rhFSH) was purchased from SERONO (Swiss), Fetal calf serum (FCS) was purchased from Gibco (UK), human chronic gonadotropin (hCG) was purchased from Organon (Holand).

Media

The handling of the harvested oocytes outside the CO_2 incubator was performed in Eagle's minimum essential medium (MEM-α) and T6 medium respectively. MEM-α medium was supplemented with 5% FCS, 6mg/ml NaHCO3, 100mIU/ml rhFSH and 7.5IU/ml hCG.

Animals and oocyte recovery

The animal studies were approved by the Razi Institute of Karaj (Iran). Female 4-6 weeks old NRMI mice were killed by cervical dislocation. Their ovaries were removed and immediately placed in pre-warmed MEM-α.
Cumulus enclosed oocytes (CEO) at GV stage were harvested by puncturing the large antral follicles with a 26-gauge needle.
Denuded oocytes were obtained by repeated pipetting of the CEO through a small bore pipette to remove the surrounding cumulus cell.

Culture of immature oocytes

Fully grown denuded oocytes at GV stage were collected and thoroughly washed three times in fresh pre-warmed MEM-α medium at 37ºC. These oocytes were then cultured in a 50µl drop of pre-incubated MEM-α under oil for IVM. This maturation medium (MEM-α) was either use as control medium (without the addition of BSO and β-mercaptoethanol) or the required amount of BSO or β-mercaptoethanol had previously been added and equilibrated in an atmosphere of 5% CO_2 in air at 37ºC for 24h before use (Figure 1).

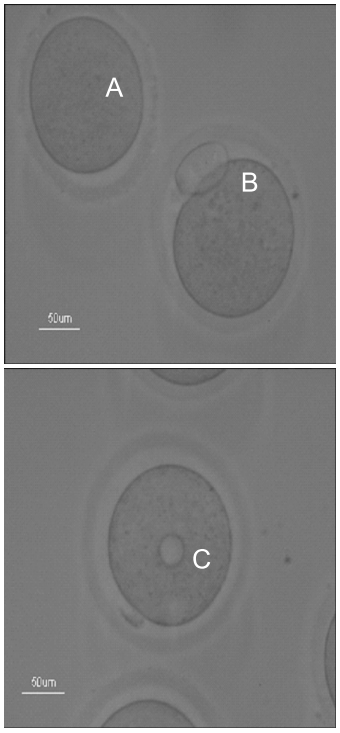

Figure 1: A: GVB, B: M II, C: GV

IVF and embryo development

Sperm were obtained from adult male NMRI mice by epididymal extraction followed by incubation for sperm was adjusted to 1×10^5 sperm/ml and then inseminated into the 50µl drop of T6 medium containing the in vitro matured oocytes. After 4-6h of insemination, the inseminated oocytes were removed from insemination medium and were cultured in groups of 5-10 oocytes per 50µl drop of T6 medium containing 4mg/ml BSA under oil at 37ºC and 5% CO_2 in air (Figure 2).

Experimental design

To study the effect of BSO and β-mercaptoethanol on nuclear maturation of denuded oocytes, denuded oocytes at GV stage were cultured for 24h either in MEM-α medium supplemented with 100µm β-mercaptoethanol (group 1), 100µm β-mercaptoethanol + 5mM BSO (group2) or MEM- α alone (control group). After 24h, the maturation stage was assessed by inverted microscope. To study the effect of BSO and β-mercaptoethanol on the fertilization and subsequent development of in vitro matured mouse oocytes, the observation of oocytes that had undergo GVBD and the formation of the first polar body were taken as signs of completion of meiotic maturation. The subsequently obtained in vitro matured oocyte was then placed in T6 medium containing of BSA 15mg/ml and inseminated with capacitated sperm for 4-6h.

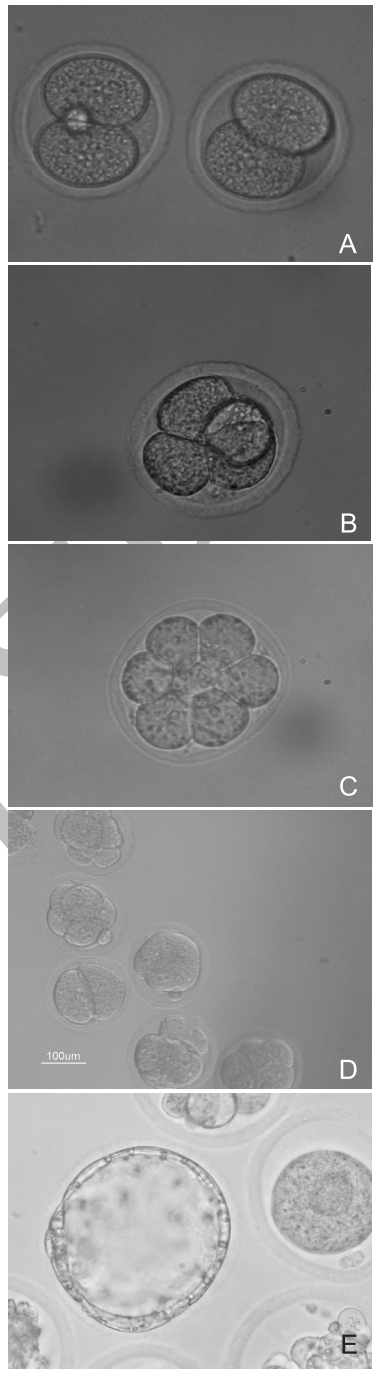

Figure 2: A: 2 cell, B: 4 cell, C: 8 cell, D: Compact morula and E: late blastocyst

These inseminated oocytes were then washed thoroughly before placing in fresh T6 medium containing of BSA 4mg/ml.

In order to investigate fertilization outcome of these in vitro matured oocytes, the inseminated oocytes were checked for the signs of fertilization (2PN) over a maximum period 4-6 h post- insemination. These with no signs of fertilization and which failed to reach the 2-cell stage after further culture were subsequently taken as unfertilized oocytes.

Table 1: Comparison in vitro maturation between experimental and control groups

Experimental Groups	No of Oocytes	BSO mM	B-mercaptoethanol µM	GV (%)	GVB (%)	MII (%)
Control	226	0	0	48 21 2	42 18 6	136 60
Group 1	262	0	100	38 14.5	40 15.2	184*a 70 2
Group 2	231	5	100	30 13	117 50.6	84*a 36.3

GV; Germinal Vesicle, GVB; Germinal Vesicle Breakdown, MII; Metaphase II
Different letters in superscript (a) within the same column are significantly different (P≤0.05).

Table 2: Comparison development matured oocytes of experimental groups

Experimental groups	Dosege Of BSO	Dosage of β-mercaptoethanol (µM)	No. of matured oocytes	after 24h insemination	after 48h insemination			after 72h insemination			after 96h insemination			after 120h insemination		
				2cell	2cell (%)	4cell (%)	Total (%)	4cell (%)	8cell (%)	Total (%)	8cell (%)	Morula (%)	Total (%)	Morula (%)	BI (%)	Total (%)
Contlol	0	0	100	43 43	36 36	16 16	52 52	25 25	5 5	30 30	3 3	5 5	8*a 8	2 2	1 1%	3*a 3
Group 1	0	100	114	54 47.3	45 46.8	21 18.4	66 57.8	21 18.4	13 11.4	34 30	3 2.6	13 11.4	16 14	14 12.2	5 4.3	19 19
Group 2	5	100	72	40 55.5	27 45.7	12 16.6	39 54	23 31.9	1 1.3	24 33.3	1 1.3	0	1*a 1.3	0*a	0	0

Values are percentages (mean) of 8 independent experiments different letters in superscript (a) within the same column are significantly different (p< 0.05)

The embryos were cultured until blastocyst stage.

Blindness

The observers were totally unaware of the mediums used for IVM and IVF for preventing any bias in their observations.

Statistical analysis

Significance of differences between the treated and control groups was analyzed by the student's T-test and one way ANOVA. Statistical significance was concluded at P<0.05.

Results

As shown in table 1, the proportion of GV oocytes maturing to metaphase I oocytes (or undergo GVBD) in control group, group 1 and group 2 were 78.5%, 85%, 86% respectively, that no significant differences between groups. The percentage of the oocytes remained at the GV stage were higher in control group than the other groups (21.2%, 14.5%, 13%).

The rate of oocytes developing to the MII stage of maturation was significantly increased with 100µM β-mercaptoethanol treatment (group 1) compared to 5mM BSO treatments (group 2) and control group (60%,70.22% 36.3% respectively) (P≤0.05).

As shown in table II, the percentage of embryo that developed to 8 cell was lower for oocytes matured in medium containing BSO in comparison to the group 1 (1.3%, 11.4%) also with BSO the percentage of embryos that developed to morula stage was significantly decreased than the group 1 and control group. The percentage of the embryo reaching to

morula stage were significantly higher in 100μM β-mercaptoethanol treatment than the control group and group 2 (5%, 12.2%, 0%) (P≤0.05). In group 2 no embryo could reach to blastocyst stage whereas in group 1 5.26% of embryos was reached to blastocyst stage.

Discussion

The result of this study indicates that the presence β-mercaptoethanol in the maturation medium positively influenced the ability of mouse oocytes to undergo meiotic maturation and resulting embryos development while presence with BSO in the maturation medium negatively effects on above roles. The percentage of oocytes develops to MII was significantly increased with 100μM β-mercaptoethanol. This percentage was significantly decreased with BSO (60%, 70.2%, 36.3% respectively).

None of embryos made of BSO treated oocytes could pass the 8 cell stage while embryos of group 1 and control group reached to the morula and blastocyst stage.

Our finding shows that β-mercaptoethanol supplementation of the IVM medium improves embryo development. BSO with β-mercaptoethanol supplementation of the IVM medium has an inhibitory effect on in vitro maturation and embryo development.

Spontaneous nuclear maturation of oocytes occurs upon liberation from follicles, which result in morphologically normal secondary oocytes (30).

However the developmental competence of these oocytes is variable because of to deficiencies in cytoplasmic maturation, even though the oocytes have a normal nuclear maturation in bovine (31-33) and pig oocytes (23). Abcydeera et al (23) demonstrated an increase in the intracellular GSH content may directly or indirectly reflect the status of cytoplasmic maturation of oocytes. The oxidative modification of cell components via oxygen species (Oxidative stress) is one of the most potentially damaging processes for proper cell function. In most cells, efficient antioxidant systems can attenuate the effect of oxidative stress by scavenging active oxygen species (37). GSH is the major non protein sulphydryl component in mammalian cells and is known to play on important role in protecting the cell from oxidative damage and also after fertilization participates in sperm decondensation parallel to oocyte activation, and in the transformation of the fertilizing sperm head into the male pronucleus (22,23,24) All of the said reports indicate that GSH increases both oocyte maturation and embryo development which is confirmed by our data. Glutathione is synthesized by the γ-glutamyl cycle (35) and its synthesis is dependent on the availability of cysteine in the medium (36). Cysteine is very unstable and rapidly oxidized to cystine in the culture medium. The lack of cysteine in the medium due to oxidation to cystine may result in GSH synthesis impairment in vitro. Low molecular weight thiols such as β-mercaptoethanol and cysteamine reduce cysteine to cysteine and promote cysteine uptake in mammalian cells (34) and therefore enhance GSH synthesis and this is probably the mechanism of what we saw in adding β-mercaptoethanol to IVM media.

Gasparrini et al (38) demonstrated that cysteamine supplementation of the IVM medium could improve embryo development via an increase of GSH synthesis. In present study the overall poor embryo development recorded in the presence of BSO demonstrated that GSH synthesis during mouse oocyte IVM is critical for embryo development as is shown in different species before (20, 21,29,38).

Conclusion

Adding β-mercaptoethanol to the IVM medium probably stimulates oocyte GSH synthesis in mouse like in other species which is necessary for mouse oocyte cytoplasmic maturation and embryo developmental rate. Moreover, the decrease in oxidative stress is probably one of the key factors responsible for the improvement of embryo development.

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بررسی تاثیر بتامرکاپتواتانول بر ازسرگیری میوز، بلوغ آزمایشگاهی و تکوین جنینی تخمک‌های نارس موش با BSO و بدون BSO

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چکیده

دریافت مقاله: ۸۴/۴/۲۶، پذیرش مقاله: ۸۴/۱۱/۱۵

* **هدف:** بررسی تاثیر بتامرکاپتواتانول بر ازسرگیری میوز، بلوغ آزمایشگاهی تخمک‌های نارس موش و تکوین جنین‌های حاصل از این تخمک‌ها با BSO و بدون BSO

* **مواد و روش‌ها:** تخمک‌های نارس برهنه (DO) از موش‌های ۶-۴ هفته‌ای جدا شده و در محیط کشت MEMα حاوی ۷/۵IU/ml hCG، ۱۰۰ mIU/ml rhfSH، ۵ درصد FCS (گروه کنترل)، ۱۰۰ میکرومتر بتامرکاپتواتانول (گروه یک)، ۱۰۰ میکرومتر بتامرکاپتواتانول و ۵ میلی‌مولار BSO (گروه دو) برای مدت ۲۴ ساعت قرار داده شدند. سپس تخمک‌های بالغ لقاح داده شده و برای ۵ روز تکوین آنها بررسی شد. لقاح و تکوین جنین در محیط T6 انجام شد.

* **یافته‌ها:** درصد تخمک‌هایی که به مرحله متافاز II(GVBD) رسیدند در گروه کنترل، گروه یک و گروه دو به ترتیب ۸۶، ۸۵ و ۷۸درصد بود که اختلاف شاخصی بین گروه‌های آزمایشی دیده نشد. نسبت تخمک‌هایی که در گروه ۲ به مرحله متافاز II رسیدند در مقایسه با گروه کنترل و گروه یک به طور شاخصی (۳۶/۳، ۷۰/۲ و ۶۰ درصد) کاهش یافت ($p\leq0.05$).
درصد متافاز II در حضور بتامرکاپتواتانول در مقایسه با گروه کنترل و گروه دو به طور چشم گیری افزایش یافت ($p\leq0.05$).
در صد جنین‌هایی که در حضور بتامرکاپتواتانول به مرحله مورولا رسیدند به طور شاخصی بالاتر از گروه کنترل و گروه یک بود. در تیمار با BSO هیچ کدام از جنین‌ها از مرحله ۸ سلولی عبور نکردند.

* **نتیجه‌گیری:** نتایج این مطالعه نشان می‌دهد که بتامرکاپتواتانول IVM را افزایش و تکوین جنینی را بهبود می‌بخشد؛ در حالی که اضافه کردن BSO به محیط کشت IVM را کاهش داده و مهار کننده تاثیرات مثبت بر تکوین جنین است.

کلیدواژگان: IVM، موش، تخمک، BSO، بتامرکاپتواتانول