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Effectiveness of Insulin Transferrin Selenium Supplementation to Vitrified Mice using Hemi Straw on Zona Hardening: Expression of Heat Shock Protein 70 and Caspase 3

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Abstract

Addition of Insulin Transferrin Selenium in vitrification medium can scavenge free radicals caused by temperatures stressors due to the freezing. Insulin Transferrin Selenium is complex protein that can stimulate cell growth, prevent cell damage due to the role of antioxidants inside, so it can maintain the viability of the embryo after thawing. Insulin Transferrin Selenium can improve the quality and viability of in-vitro blastocyst culture results. The purpose of this study is to prove the effectiveness Insulin Transferrin Selenium supplementation in vitrification medium can reduce the hardening zone of mouse embrio post thawing, proving the effectiveness of Insulin Transferrin Selenium supplementation in vitrification medium on the expression of Heat Shock Protein 70 in mouse embryos post thawing, and prove the effectiveness of Insulin Transferrin Selenium supplementation the vitrification medium can decrease the expression of caspase 3 in mouse embryos post thawing. This study covered the stages of superovulation and collection eggs, in-vitro fertilization (IVF), modification vitrification medium with supplementation of Insulin Transferrin Selenium (ITS), embryo vitrification with hemi straw, embryo culture, examination of the hardening zone of mouse embryo, examination of Heat Shock Protein 70 expression and examination of caspase 3 expression in embryos post thawing. The results showed that there were differences between the addition or supplementation of Insulin Transferrin Selenium and without addition of Insulin Transferrin Selenium in the vitrification medium to hardening zone and caspase 3, but it was no differentiation in the expression of Heat Shock Protein 70. The conclusion is the supplementation of Insulin Transferrin Selenium in vitrification medium decreases the hardening zone of the mouse embryos post thawing, decreases the caspase 3 expression in the mouse embryos post thawing and there is no difference in the expression of Heat Shock Protein 70 in the mouse embryos post thawing.

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Introduction

Vitrification method is where material is frozen and placed in hyperosmolarity media or media with a high concentration of cryoprotectant. Then the material is directly immersed in liquid nitrogen so that the frozen solution seems to be as glass. Technically, this method can reduce cell damage due to freezing temperatures. In addition this method can reduce embryo damage due to freezing temperatures because the critical temperature can be exceeded by very fast and can avoid the formation of ice crystals that can damage the cells[1,2].

Vitrification method has some problems, which make declining quality of embryos post thawing due to the thickening of pellucida zone. The thickening of pellucida zone will affect the attachment of the embryo to endometrium at the time of implantation. Hardening zone will affect the attachment of endometrium tropoblast while implantation[3]. Loss of viability of embryos also caused many blastomere cells under going apoptosis as a result of the low temperature stressor to high temperature while thawing. Improvement of Heat Shock Protein70 during freezing will result in increased caspase 3 that trigger cell apoptosis.

A decrease in viability of embryo post thawing greatly affects to the successfull rate of embryo implantation, more over will decrease the pregnancy rate. This is possible, because the formation of hardening zone or thickening of pellucida zone in frozen embryos after thawing, making it difficult for implantation. Therefore,a study is required to optimize the vitrification medium, in order to optimize the role of cryoprotectant to protect the embryo from temperature stressors of vitrification method. Addition of Insulin Transferrin Selenium in vitrification medium can scavenge free radicals caused temperature stressors due to freezing[4]. Insulin Transferrin Selenium is a complex protein that can stimulate cell growth, prevent cell damage due to the role of antioxidants inside, so it can maintain the viability of embryos post thawing. According to[3], Insulin Transferrin Selenium can improve the quality and viability of in-vitro blastocyst culture results.

Based on the background, research is needed to prove the effectiveness of Insulin Transferrin Selenium on the thickness of the pellucida zone, Heat Shock Protein 70 and Caspase3 in the mouse embryos vitrified with hemistraw.

Materials and Method

This study was experimental laboratory and the design was Completely Randomized Design (CRD), assuming all treatments from sampling to implementation as well as laboratory conditions were same.

Superovulation and collection of eggs

Female mice were injected with Pregnant Mare Serum Gonodotropin (PMSG; Foligon, Interved) at dosage of 5 IU. Forty-eight hours later they were injected with Human Chorionic Gonadotropin (HCG or Chorulon) and directly mated with castrated male monomattingly. Seventeen hours later, the vaginal plug was examined. Female mice with positive vaginal plug means that the egg can be collected. Then the female mice with positive vaginal plug, to removed and collected the fallopian tubes. Furthermore, the fallopian tubes were flushed with PBS in the petri dish, and examined under inverted microscope by tearing fertilization bags. Finally,the flushed eggs were washed and prepared for *in-vitro* fertilization.

In- Vitro fertilization

The collected eggs were washed three times in PBS and MEM medium. The washed eggs were transferred to the medium of fertilization. Spermatozoa were taken from cauda epididymal of male mice, then immersed in the fertilization medium where the eggs already in it. The eggs were mixed with spermatozoa then incubated in 5% CO_2 incubator at a temperature of 37°C for 7 hours, then the granulosa cells were dropped to observe 2 pn.

Culture of embryos to reach morula stage

Once 2 pn was formed, the zygote was transferred into culture medium and incubated in 5% CO_2 incubator at 37°C. Culture medium was replaced every 2 days until the embryos reached the morula stage.

Modification of the vitrification medium with supplementation Insulin Transferrin Selenium(ITS)

The vitrification medium contain PhosphateBufferSaline(PBS) added with intracellular cryoprotectant Ethylenglycol (EG) 30% and Sucrose1M. The modification medium was the same as mentioned above but it was added by adding Insuline Transferrin Selenium with dosage of 5 µg/ml, 10 µg/ml and15 µg/ml[5]. So overall this study consist of 4 groups. Those groups were control group (PBS+30% EG+Sucrose1M), group1 (PBS+30% EG+Sucrose1M+ITS5 µg/ml, group2 (PBS+30% EG+Sucrose1M+ITS10 µg/ml) and group3 (PBS+30% EG+Sucrose1M+ITS15 µg/ml).

Embryo vitrification with hemi straw

This vitrification used[6] modified method. Morula embryo stage entered to the vitrification medium 1containing PBS for 2 minutes, then transferred to vitrification medium 2 containing 30% EG+ITS for 2 minutes, then transferred medium 3 containing 1M sucrose for 30 seconds, then the embryo was place that the end of the hemistraw. Furthermore hemistraw that has been revealed by liquid nitrogen dipped in liquid nitrogen and put in a largestraw, so the embryo will be remain at the end of the hemi straw. Then the big straw fixed on each tips and inserted into the cassette straw. Furthermore cassette inserted in container goblet of liquid nitrogen.

Thawing frozen embryos

Thawing embryos used[6] modified method. Embryo was removed from the straw, then dropped into the vitrification medium 4 containing PBS+1M sucrose for 2 minutes, then transferred to medium vitrification 5 containing PBS+0.5 M sucrose for 2 minutes, then transferred to PBS medium for 2 minutes. Furthermore morula embryos cultured in $C0_2$ incubator for 24 hours to observe the development of embryo searching the blastocyst stage.

Examination of hardening zone

Examination of hardening zone was performed with image moticprograme using inverted microscope. Measurements were performed 3 times for each embryo, then the results of the thickness zone is the average value of the measurement results.

Examination of heat shock protein 70 expression by immunocytochemistry

After thawing embryos were fixed on objects glass, then rehydrated with multi level alcohol concentration, washed with PBS, and then soaked respectively in 3% hydrogen peroxide (in DIwater) for 20 minutes, 1% BSA in PBS 30 minutes at room temperature, Primary Antibodies (anti-HSP 70) 1: 1000 overnight at 4°C, biotin-labeled secondary antibody (anti-Rat IgG Biotin Labelled) and primary antibody anti-HSP 70, 1 hour

at room temperature, the SA-HRP (Sterp Avidin-Hoseradish Peroxidase), 60 minutes at room temperature, chromogen DAB (3, 3-diaminobenzidine tetrahydrochloride), 20 min atroom temperature, counter stain (Acetoorcein), 3 minutes at room temperature and finally examined under a light microscope.

Examination of the expression of caspase3 with immunocytochemistry

After thawing embryos were fixed on objects glass, then rehydrated with multi level alcohol concentration, washed with PBS, and then soaked respevtively in 3% hydrogen peroxide (in DIwater) for 20 minutes, 1% BSA in PBS 30 minutes at room temperature, Primary Antibodies (anti-HSP 70) 1: 1000 overnight at 4°C, biotin-labeled secondary antibody (anti-Rat IgG Biotin Labelled) and primary antibody anti-HSP 70 1 hour at room temperature, the SA-HRP (Sterp Avidin-Hoseradish Peroxidase) 60 minutes at room temperature, chromogen DAB (3, 3-diaminobenzidine tetrahydrochloride), 20 minutes atroom temperature, counter stain (Acetoorcein) 3 minutes atroom temperature and finally examined under a light microscope.

Data Analysis

The data was conducted in a same controlled environmentand conditions. Data obtained from the picture of hardening zone, the expression of HSP70, Caspase 3 was tested by ANOVA and if there is a difference between treatment followed by Duncantest[6].

Results

Viability of mouse embryos after thawing

The viability of the thawed embryos have tested by ANOVA, and showed significant difference ($p < 0.05$), followed by the Tukey test to see the difference in each treatment. The results can be seen in (Table 1).

Table 1: Average Viabilityof Mouse Embryos after Thawing

Treatment Group	X ± SD
Control group	4.33 ^{a} ± 1,2
Treatment group 1	5.00 ^{a} ± 1,8
Treatment group 2	7.33 ^{b} ± 0,5
Treatment group 3	7.56 ^{b} ± 0,7

Notes : different superscripts in the same column is significaly different (p<0,05)
Controlgroup : PBS + EG 30 % + Sukrosa 1 M
Treatment group1 : PBS + EG 30 % + Sucrose 1 M + ITS 5 µg/ml
Treatment group 2 : PBS + EG 30 % + Sucrose 1 M + ITS 10 µg/ml
Treatment group 3 : PBS + EG 30 % + Sucrose 1 M + ITS 15µg/ml

Insulin Transferrin Selenium was given in vitrification medium could increase the viability of mouse embryos after thawing. It can be seen from the results of the analysis between the control group without Insulin Transferrin Selenium and the treatment with Insulin Transferrin Selenium. The viability of the mouse embryos after thawing in the control group was not significantly different from the group treated with the addition of Insulin Transferrin Selenium with a dosage of 5 µg/ml, but there is significantly differentin dosage of 10 µg/ml and 15 µg/ml Insulin Transferrin Selenium group respectively. Moreover, the addition of Insulin Transferrin Selenium with a dosage of 5 µg/ml was significantly different from the group treated with the addition of Insulin Transferrin Selenium with a dosage of 10 µg/ml and 15 µg/ml, but the group treated with addition of Insulin Transferrin Selenium with a dosage of 10 µg/ml and 15 µg/ml were not significantly different. The results of statistical tests in the (Table1) showed that the addition of Insulin Transferrin Selenium with a dosage of 10 µg/ml and 15 µg/ml contributed the best results of the mouse embryo viability after thawing.

Hardening zone of the mouse embryos after thawing

Based on the results of the calculation on the thickness of pellucida zone (hardening zone) on the embryos after thawing, tested by ANOVA, showed significant difference ($p < 0.05$), followed by Duncan test to see the difference among the treatment. The results showed in (Table 2).

Table2: Mean and standard deviation of the thickness of the pellucida zone on mouse Embryos after thawing

Group	Mean ± SD
Control group	14,53^{b} ± 1,72
Treatment group 1	14,37^{b} ± 1,83
Treatment group 2	13,70^{ab} ± 1,98
Treatment group 3	12,67^{a} ± 1,57

Notes : different superscript in the same column is significant different ($p < 0,05$)
Control group : PBS + EG 30 % + Sukrosa 1 M
Treatment group1 : PBS + EG 30 % + Sucrose 1 M + ITS 5 µg/ml
Treatment group 2 : PBS + EG 30 % + Sucrose 1 M + ITS 10 µg/ml
Treatment group 3 : PBS + EG 30 % + Sucrose 1 M + ITS 15 µg/ml

The ANOVA analysis on the thickness of the zone above showed $F_{hitung} = 3.362$ with significance of 0.025 <0.05, so there is a notice able difference intreatment, further more testing was done with Duncan test in order see the difference in each treatment. The hardening zone control group was not significantly different compared with the group added with Insulin Tranferrin Selenium with a dosage of 5 µg/ml and 10 µg/ml, but significantly different from the treatment group with Insulin Transferrin Selenium dosage of 15 µg/ml. Moreover, the addition of Insulin Transferrin Selenium with a dosage of 15 µg/ml was not significantly different from the group treated with the addition of Insulin Transferrin Selenium with a dosage of 10 µg/ml, but significantly different from the group treated with the addition of Insulin Transferrin Selenium with a dosage of 5 µg/ml. The statistical tested resulting the (Table 2) showed that the addition of Insulin Transferrin Selenium with a dosage of 15 µg/ml gave the best results due to reducing of hardening zone, so the embryos can hatch and implant.

Expression of Heat Shock Protein70 (HSP 70) in mouse embryos after thawing

Based on the calculation of HSP 70 expression of mouse embryos after thawing, was tested using Kruskall-Wallistest, the result showed not significantly ($p > 0.05$). The result shows in the (table 3) below. The result of Kruskall-Wallistest on HSP 70 data showed not significantly of 0.919 > 0.05, means there was not significantly difference on the treatment. Therefore

it was not continue with Mann-Whitneytest. HSP 70 expression of mouse embryos after thawing in the control group was not significantly different from treated with the addition of Insulin Transferrin Selenium with a dosage of 5 µg/ml, 10 µg/ml, and 15 µg/ml. Similarly, the addition of Insulin Transferrin Selenium showed no significant difference among treatment groups. The ineffectiveness of the addition Insulin Transferrin Selenium to vitrification medium on the expression of Heat Shock Protein 70 in the mouse embryos in post thawing was probably there is no cold shock during post thawing. The increased expression of Heat Shock Protein 70 during the vitrification to protect against cold shock.

Tabel 3: Results of statistic analysis HSP 70 expression of mouse embryos after thawing

Group	Mean	Asymp. Sig.
Control group	177,50[a]	0,919
Treatment group1	177,50[a]	
Treatment group 2	181,50[a]	
Treatment group3	185,50[a]	

Notes : Same superscript in the different column is not significant (p>0,05)
Control group : PBS + EG 30 % +Sukrosa 1 M
Treatment group 1 : PBS + EG 30 % + Sucrose 1 M + ITS 5 µg/ml
Treatment group 2 : PBS + EG 30 % + Sucrose 1 M + ITS 10 µg/ml
Treatment group 3 : PBS + EG 30 % + Sucrose 1 M + ITS 15 µg/ml

Expression of caspase 3 in mouse embryos after thawing

Based on the calculation of caspase 3 expression of mouse embryos after thawing, tested using Anova test, the result obtained was significantly very different ($p < 0.05$), then continued with Tukey test to find out differences of each treatment. The result shows in the (table 4).

Table 4: Average and standard deviation of expression of caspase 3 in mouse embryos after thawing

Treatment Group	X ± SD
Control group	5.89 [a] ± 1.2
Treatment group 1	5.11 [ab] ± 1.7
Treatment group 2	4.00 [bc] ± 1.2
Treatment group 3	3.22 [c] ± 0.9

Notes : different superscript in the same coloumn is significantly different ($p < 0.05$)
Control Group : PBS + EG 30 % + Sucrose 1 M
Treatment Group 1 : PBS +EG 30 %+ Sucrose 1 M + ITS 5 µg/ml
Treatment Group 2 : PBS + EG 30 %+ Sucrose 1 M + ITS 10 µg/ml
Treatment Group 3 : PBS + EG 30 % + Sucrose 1 M + ITS 15 µg/ml

Data showed significance of 0.01< 0.05, therefore there was significantly difference on the treatment. Further test using Tukey test was carried out, to see the difference of each treatment. The expression of caspase 3 in mouse embryos after thawing of control group was not significantly different from that of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 5 µg/ml dan 10 µg/ml, but it was significantly different from that of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 10 µg/ml and that 15 µg/ml respectively. Moreover, the expression of caspase 3 in mouse embryos after thawing of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 5 µg/ml was not significantly different from that of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 10 µg/ml, but it was significantly different from that of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 15 µg/ml. Moreover, the expression of caspase 3 in mouse embryos after thawing of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 10 µg/ml was not significantly different from that of treatment group supplemented with Insulin Transferrin Selenium with a dosage of 15 µg/ml.

Discussion

Viability of mouse embryos after thawing at least determined the success rate of embryo transfer and their of pregnancy, however many factors can influence the rate of pregnancy, such as hormonal condition in recepient, reproductive tract condition of the recipient, implantation success, and other factor.

The result showed adding Insulin Transferrin Selenium with a dosage of 10 µg/ml and that of 15 µg/ml gave the best result becuse it could decrease apoptosis level of mouse embryos post thawing, therefore when the mouse embryos were re-cultured and the transfer of mouse embryos was done, it was expected successfull rate of embryo growth will be very high. The decrease of caspase 3 related to blastomer cell vitality would increase the viability of the mouse embyos after in-vitro culture, therefore it could increase the success rate of embryo transfer and their pregnancy. Invitro culture as culture conditions, the addition of cells to the media, hormone supplements and additional growth factors not only affect embryonic development but also the viability of embryos after freezing and warming[7-9]. In addition, the quality and stage of embryo development is also important for survival after freezing and thawing[10]. The types of factors cryprotectant, freezing, and thawing procedures can also affect the viability of embryos after thawing. Glycerol a cryoprotectant is better than the Ethylene glycol on the quality of the blastocyst embryos after thawing[11]. According to[12], states that embryo viability after cryopreservation correlated with the level of development of the early embryo frozen[13]. Reported the viability embryo morula after frozen is lower than blastosis. Embryonic stage development very sensitive to the freezing and affect the viability of embryos after thawing[11].

The addition of ITS on the freezing spermatozoa can improve sperm motility post-thawing. AFP can act through mechanisms non colligative to inhibit the growth of ice crystals, with thermal hysteres is (lowering the freezing point locally, creating a difference between freezing and melting point), or by interacting directly with the membrane and increase the stability of phospholipids membrane[4]. Freezing and thawing back proved can cause significant changes in lipid peroxidation in human sperm membrane, causing damage morphology, viability and decreased motility[14]. Addition of Insulin Transferrin Selenium in vitrification medium possible because Insulin Transferrin Selenium binded free radicals through increased glutathione[15,16]. Free radicals are molecules with no electron pairs and are highly toxic to cells as they will damage endogene anti-oxidants, consequently it will decrease glutathione peroxidase. The decrease of

glutathione would cause cell damage due to the increase of poly unsaturated fatty acid (PUFA) and the result of the peroxidation lipid process,in the form of Malondeadehid binded with components in cell membranes which were able to damage structure and function of cells[17,18]. Selenium content of the ITS will be conjugated with glutathione peroxidase and catalase containing iron. Transferrin is considered able to protect cell membranes against peroxidation caused by free radicals.

Cell damage due to oxidative stress is related to mitochondria damage, which will released pro-apoptosis molecules. When permeability of mitochondria membranes were damaged, anti-apoptosis molecules in mitochondria membraneswere replaced by pro- apoptosis ones and the condition would activate caspase 3 as executorof apoptosis[17]. Oxidative stress originated from vitrification process of Reactive Oxygen Spesien (ROS) caused by cold shock. The decrease in embryo viability showed that cryoprotectant was unable to protect embryos optimally due to the oxidative stress. Adding Insulin Transferrin Selenium was able to scavenge free electrons released on targetted cells, therefore it would increase glutathione. The glutathione increase would muffle ROS released during cold shock, therefore it would get better embryo viability.

Hardening zone could decrease the rate of implantation as trophoblast cells and inner cell mast can not get out of pellucida zone, therefore trophoblast could not stick on endometrium of uterus, consequently implantation process would not be happened. The decrease in pellucida zone thickness in this study needs to be proven further if it is equivalent with the increase in pregnancy rate if the embryo from thawing is transferred to recipient. HSP 70 was always found in the basal condition or a condition without stress, but the expression of HSP 70 increased when the cells were under stress. In the embryo vitrification cold shock occurred. The very drastic changes in temperature cause and increase in HSP because HSP 70 properties as chaperone molecules, that is, directing proteins involved in the process of synthesis, translocation and prevention of aggregation[19]. In post thawing HSP70 expression did not differ between the groups with and without supplementation of Insulin Transferrin Selenium. This possiblility due to the absence of cold shock, so HSP 70 did not play a role in the process of protein denaturation induced by temperature stress, so there was no increase in the expression of HSP 70.

Conclusion

The conclusion of this study is that Insulin Transferrin Selenium in the vitrification medium decreases hardening zone and caspace 3 expression in mouse embryos after thawing, but there is no difference in Heat Shock Protein70 expression between the groups and without supplementation of Insulin Transferrin Selenium respectively.

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