Effect of Combined Cryoprotectant of Ethylen Glicol and Propanodiol on Embryo Cryopreservation to Blastomere Cell Apoptosis and Blastocyst Quality

Epy Muhammad Luqman[1], Widjiati[1], Suryo Kuncorojakti[1]*

1. Department of Veterinary Anatomy t, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Abstract

Freezing embryo is a method to store embryo. So far embryo quality after it is frozen then warmed is still low, therefore when the embryo is transferred to recipient; it will result in low conception rate. Use of single cryoprotectant is not able to maximally protect embryo to extreme temperature change, it is shown on post warming embryo quality which is still low. Use of combined cryoprotectant of ethylene glicol and propanediol in order to maximally protect intracellular embryo as both cryoprotectants have different characteristics to protect cell.

To investigate compositions of cryoprotectant medium which is able to maximally protect embryo so that it results in high conception rate post warming.

The research was divided into four groups: T1 : Etylene Glicol 30%, T2 : Propanediol 30%, T3: Etylene Glicol 10% + Propanediol 10%, T4: Etylene Glicol 15 % + Propanediol 15%. Freezing embryo was done for a week then warming was carried out, next examination on viability and apoptosis of blastocyst was done.

Blastocyst viability of T4 was the highest compared to the other groups (82.75± 4.944; $p<0.05$). Observation on blastomere apoptosis showed that blastomere apoptosis of group T3 (7.20 ± 2.168; $p<0.05$) and T4 (4,80 ± 1,304; $p<0.05$) was lower than that of group T1 and T2. Combination of Etylene Glicol 15% + Propanediol 15 % was the best cryoprotectant to increase blastocyst viability and decrease number of apoptosis.

Experimental article (J Int Dent Med Res 2017; 10(3): pp. 1074-1079)

Keywords: Embryo, Vitrification, Blastocyst Viability, Warming, Cryopreservation.

Received date: 24 August 2017 **Accept date:** 10 October 2017

Introduction

Freezing embryo or cryopreservation is a method to store excessive embryos resulted from in vitro fertilization.[1] Excessive embryos can be stored and later at the right time, they can be transfer back to recipient.[2,3,4]Excessive embryos are highly expected by patients who are having test tube baby program. It is hoped that when implantation fails, there are still embryos left which are stored without going through long test tube baby process so that embryo resulted from freezing process can be transferred.[5,6]

Method which is developed as embryo cryopreservation is using vitrification method.[7] Vitrification method is done by freezing embryo fast at low temperature (-196ºC) using high concentration cryoprotectant is intended to prevent from ice crystals formed which is able to damage blastomere cell, ice crystals formed during freezing will damage embryo damage organelle in cyst mitochondria and induce cell lose membrane plasma integrity.[8] Cell damage resulted from ice crystals formed is able to lead to cell death of embryo.[9]

Cryoprotectant is needed to protect embryo during cryopreservation process. Cryoprotectant functions to protect embryo from drastic temperature change that is from warm temperature to minus temperature and on the other hand, during warming from minus temperature to warm temperature.[10] Cryoprotectant used so far is intracellular single cryoprotectant that is only ethylene glicol or only propanediol which works to protect embryo from drastic temperature change so that it is not able to maximally protect embryo seen from low quality embryo post warming.[11,12] During

Corresponding author:
Dr. Widjiati, DVM. M.Sc.
Department of Veterinary Medicine,
Faculty of Veterinary Medicine,
Universitas Airlangga. Surabaya, Indonesia.
E-mail: widjiati@fkh.unair.ac.id

cryopreservation and warming process, cell will experience damage (apoptosis) as a result of a very low temperature exposure -196^{0}C, it is likely to form ice crystals, free radicals due to temperature shock and rehydration.[13] Combined cryoprotectant ethylene glicol dan propanediol is intended to maximally optimize intracellular cryoprotectant by intracellularly protecting embryo as both cryoprotectants have different characteristics to protect cell during freezing process.[7,14]

According to the research by Somfai et al., (2015) use of double cryoprotectants with respective combined cryoprotectants with right embryo growth for transfer.[15] Cryopreservation using double cryoprotectants is able to increase embryo viability after warming or in vitro culture.[16] Low embryo viability will influence implantation that is adhering embryo to endometrium. Decreased embryo quality and viability is also caused by a number of trophoblast cells which function as embryonic placenta which experiences apoptosis, consequently implantation and gestation do not exist. Besides, endometrium thickness of recipient must be ready to receive embryo.[17,18,19]

Embryo viability post warming highly influences implantation rate and gestation after embryo is transferred to recipient. Therefore, study is needed to optimize cryoprotectant medium so that it is able to embryo quality after warming and decrease blastomere cell apoptosis. The research is intended to produce composition of cryoprotectant medium which is able to maximally protect embryo during freezing process and produce embryo with high viability post warming.[20,21]

Materials and methods

Research Ethical Clearance

This research received ethical clearence number: 717-KE released by Animal Care and Use Committee, Airlangga University, Faculty of Veterinary Medicine.

Research Design

Research design used was complete random design. With this design, the source of variability is only treatment. Besides treatment, other variables are homogenous.

Materials and Research Equipments

Materials used in the research were five month old male rats (Pusvetma Surabaya), three month old female rats (Pusvetma Surabaya), Pregnant Mare Serum Gonodotropin (PMSG) (Folligon®, Intervet, Boxmeer, Holland), Human Chorionic Gonadotropin (HCG) (Chorulon®, Intervet, Boxmeer, Holland), Phosphate Buffer Saline (PBS), Medium Engle Minimum (Sigma®, St. Louis, USA), ethilen glikol (Sigma®, St. Louis, USA), propanediol (Sigma®, St. Louis, USA), mineral oil (Sigma®, St. Louis, USA), gentamycin sulfat, CO_2.

Equipments used in the research were CO_2 incubator (Thermo Fisher Scientific), inverted microscope (Meiji Techno America), image raster program 2 .2, syringe, pipet pasteur, Hemi straw (Sigma®, St. Louis, USA), dispossible petridish (Thermo Fisher Scientific), millipore (Thermo Fisher Scientific).

Superovulation and egg cell collection

Female rat of BALB/c strain, weighing 30-35grams, three months old, healthy, active, never used for research was injected using hormone of *P*regnant Mare Serum Gonodotropin (PMSG atau Foligon) with the dosage of 5 IU. 48 hours later it was injected with hormone of *H*uman Chorionic Gonadotropin (HCG atau Chorulon) and directly mated with male rate of BALB/c strain weighing 40-45grams, five months old which was monomattingly castrated. Seventeen hours after female rat was mated, vagina plug examination was conducted. Egg cell collection was done on female rat with its vagina plugged. Then, it was decapitated, cut, and its fallopian tube was taken out. Next, fallopian tube was washed with solution of *P*hosphate Buffer Saline, after that, moved to petridish and flushed under inverted microscope by ripping fertilization pouch. merobek kantong fertilisasi. Finally, flushed egg cell was washed and prepared for in vitro fertilization.

In vitro fertilization

Collected egg cell then was washed three times respectively in medium of PBS and MEM. Washed egg cell was next moved to fertilization medium. To wait until spermatozoa prepared for in vitro fertilization. Spermatozoa was taken from cauda epididymis of male rat, then soaked in fertilization medium with egg cell in it. Egg cell which was mixed with spermatozoa

was incubated in CO_2 incubator of 5% with temperature of 37° C for 7 hours, then granulosa cell was threshed to observe 2 pn (Beyer and Griesinger, 2016).

Embryo culture until morula stage

After 2 pn was formed, then zygote was moved to culture medium and incubated in $C0_2$ incubator of 5% at temperature of 37°C. Culture medium was changed once in two days until embryo reached morula stage.

Embryo cryopreservation was done by using vitrification method and combined cryoprotectant. Overall the research consisted of 4 groups with each group consisting of five rats: Treatment Group 1 (T1) : Etylene Glicol 30 %; Treatment Group 2 (T2) : Propanediol 30 %; Treatment Group 3 (T3) : Etylene Glicol 10 % + Propanediol 10 %; Treatment Group 4 (T4) : Etylene Glicol 15 % + Propanediol 15%. Embryo expossed with cryoprotectant medium of ethylene glicol and propanediol, then was put in the tip of hemi straw. Next, hemi straw expossed with liquid N2 was dipped into liquid N2 and put into big straw. Putting hemi straw into big straw must be done in liquid N2, so that embryo at the tip of straw was not gone. After that, tip of big straw was fixed and put into straw cassette. Finally, straw cassette was put into goblet container of liquid N2.

Warming embryo

Before warming, medium consisting of V4 (PBS medium + Sucrose 0,5 M), V5 (PBS medium + Sucrose 1 M) was warmed for 15 minutes. Embryo after warming was put into medium V4 for 2.5 minutes, then moved to medium V5 for 7.5 minutes. Next, before embryo was tranferred, embryo was incubated in $C0_2$ incubator for 2 hours.

Apoptosis examination using immunocytochemistry method

Embryo at blastula stage was fixed on glass object, then rehydration was done with level alcohol, next washed with PBS, after that, soaked in 3% hydrogen peroxide H_2O_2(in DI water) for 20 minutes, , 1% BSA in PBS for 30 minutes at room temperature. , Apoptag kit (TACS® 2 Tdt DAB *In situ* Apoptosis Detection Kit, TREVIGEN® inc., Maryland) 1:1000 for an hour, cold temperature of 4°C, Secondary antibody biotin labelled (*Anti Rat IgG Biotin Labelled*) and primary antibody of Apoptag kit, 1 hour at room temperature, , SA-HRP (Sterp Avidin- Hoseradish Peroxidase), 60 minutes, room temperature , Cromogen DAB (3,3-d*iaminobenzidine tetrahydrochloride*), 20 minutes, room temperature , Counterstain (methil green), 3 minutes, room temperature then was checked under microscope. Every stage changed, it had to be washed with PBS to clean it from other materials sticking on it.

Data Analysis

Data analisys used data of *O*ne Way ANOVA (Analysis of Variance). Data was processed using program of SPSS 20 (Statistical Package for Social Science), Chicago. USA. If there was significant difference among treatment groups, Duncan test was carried out.

Results

Blastomere Cell Apoptosis

Based on result of treatment on various groups, number of blastomere cell apoptosis was able to be counted. Data taken was then tested to find out normality and homogeneity using Kolmogorov-Smirnov test and Shapiro-Wilk test. Number of blastomere cell apoptosis was next tested using *O*ne Way ANOVA and if there was significant difference ($p<0,05$) , Duncan test was carried out.

Result of test on number of blastomere cell apoptosis using *O*ne Way ANOVA showed F count= 13.905 with significant difference ($p<0,05$). Therefore, Duncan test was conducted to find out difference of each group treatment. Result of Duncan test can be seen in the table 1 below.

Treatment Group	Number of blastomere cell apoptosis (X±SD)
Treatment 1 (T1)	$11.20^{b} \pm 3.564$
Treatment 2 (T2)	$13.60^{b} \pm 1.817$
Treatment 3 (T3)	$7.20^{a} \pm 2.168$
Treatment 4 (T4)	$4.80^{a} \pm 1.304$

Note: Different superscript in the same coloumn shows significant difference ($p<0,05$)

Table 1. Average and standard deviation of number of blastomere cell apoptosis of treatment groups with different cryoprotectant.

Result of Duncan test on number of blastomere cell apoptosis showed that group of Etylene Glicol 10 % + Propanediol 10 % (T3) and

an Etylene Glicol 15 % + Propanediol 15 % (T4) had significant difference if compared to group of Etylene Glicol 30 % (T1) dan Propanediol 30 % (T2).

Result of the research showed that use of Etylene Glicol dan Propanediol combined as cryoprotectant had significant difference to decrease number of blastomere cell apoptosis(Figure 1,2,3).

Blastocyst Quality

Result on treatment on various groups yielded blastocyst quality data. Evaluation on blastocyst quality blastocyst quality was done by counting blastocyst variable after vitrification was done. Data taken was then tested to find out normality and homogeneity using Kolmogorov-Smirnov test and Shapiro-Wilk test . Number of blastocyst variable after vitrification was next tested using *One Way* ANOVA and if there was significant difference, Duncan test was carried out.

There was significant difference among treatment groups. Result on variant analysis showed that F count = 20.914 with significant difference ($p<0,05$). Duncan test was carried out as further test to find out difference of each treatment group. Duncan test result can be seen in table 2 below.

Treatment Group	Blastocyst Viability Percentage (X±SD)
Treatment 1 (T1)	$64.24^{a} \pm 5.283$
Treatment 2 (T2)	$60.48^{a} \pm 5.537$
Treatment 3 (T3)	$75.01^{b} \pm 3.966$
Treatment 4 (T4)	82,. ± 4.944

Note:Different superscript in the same coloumn shows significant difference ($p<0,05$)

Table 2. Average and standard deviation of blastocyst viability percentage of treatment groups with different cryoprotectant.

Duncan test result on blastocyst viability percentage showed that group of T3 and T4 had significant difference to group of T1 and T2.Group T4 had the most difference compared to the other treatment groups with notation c. Group T2 had significant difference with group T1 and T2 with notation b.

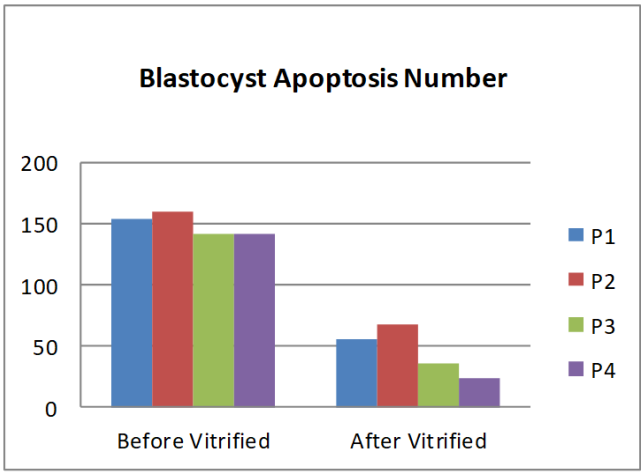

Figure 1. Blastocyst Apoptosis Number of each group.

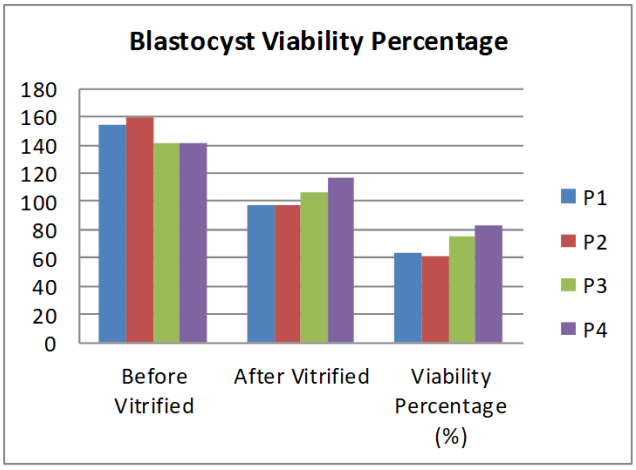

Figure 2. Blastocyst Viability Percentage of each group.

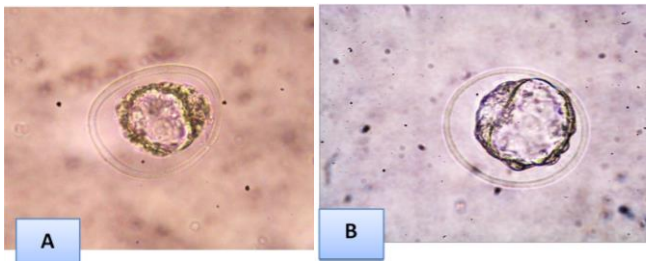

Figure 3. Figure A shows embryo which experiences degeneration. Figure B shows living embryo.

Discussion

Cell experiences physical stress at cryopreservation procedure, several cells are able to tolerate stress better than other cells, and change on cell membrane is able to induce change on cell to tolerate with stress. Main stress

is osmotic change, cell and cell parts experience big change on volume due to movement of water and intracellular cryoprotectant. Further, cell with flexible membrane has less damage compared to cell with rigid membrane. Moreover, the higher permeability of cell membrane to water and cryoprotectant, the less osmotic stress.[22,23]

Main components of cell membrane are phospholipid, cholesterol. Other lipids and protein. Except protein, these components are able to be manipulated in various ways such as, nutritional status and composition of culture medium. Overall, these components are necessary in the form of liquid, solid or gel. Some areas of cell membrane are ready for fluidity change to be gel during temperature change. Change that takes place in this phase is irreversible, during warming cellular components are not able to be reunited. The best choice to eliminate transition from liquid to be gel perfectly at freezing process; another strategy used is allowing transition to take place at low temperature or accelerating transition time to decrease changes that are able to damage cell component.[7]

Slow-rate freezing is intended to balance various factors that are able to cause damage such as ice crystals formed, fractures, toxics, and osmotic change. Controlled freezing rate leads to liquid change intracellularly and extracellularly without serious osmotic effect and change of cell shape (equilibrium freezing).[24,25]

Cryoprotectant concentration is high at final phase, if toxic effect is able to be minimalized. Intracellular ice formation is able to be reduced and it almost does not exist. Phenomenon of freezing water without forming crystals is called vitrification. Vitrification in cryobiology refers to cryopreservation method which has main purpose to make sure that ice crystals do not exist. Vitrification has higher vialibility rate after warming compared to Slow Cooling Freezing.[5,26,27]

Various researches keep being done to decrease toxicity of cryoprotectant by modifying chemicals and using more permeable chemicals (like etylene glicol), using two or three cryoprotectants to reduce toxic effect of each cryoprotectant.[13,28] Low embryo viability after freezing is by modifying concentration and type of cryoprotectant, exposure time of different cryoprotectant, procedure temperature, use of additional materials such as sweets or surfactant.[10]

Vitrification needs high concentration cryoprotectant. Therefore, it is necessary to minimalize cell damage due to osmotic stress or chemicals toxicity . cryoprotectant prevents from ice crystals formed which induce main damage, but use of high concentration cryoprotectant is able to be toxic and induce osmotic damage. Various methods used to find out ideal cryoprotectant : reduction of exposure time of cryoprotectant, use of cryoprotectant which has low toxicity, combination of several cryoprotectants, cryoprotectant exposure to low temperature.[7] The research found out that use of combined cryoprotectant etylene glicol dan propanediol was able to increase blastocyst quality and decrease blastomere cell apoptosis.

Conclusions

Combination of etylene glicol and propanediol was able to decrease blastomere cell apoptosis and increase blastocyst viability as parameter of blastocyst quality. Combined dosage of etylene glicol 15% and propanediol 15% was the best dosage to decrease blastomere cell apoptosis and increase blastocyst viability.

Acknowledgement

The authors wish to thank Faculty of Veterinary Medicine, Universitas Airlangga.

Declaration of Interest

The authors declare no conflict of interest.

References

1. Xu, B. Y. He. Y. Hong. Y. Wang. Y. Lu. Y. Sun. Frozen embryo transfer or fresh embryo transfer: Clinical outcomes depend on the number of oocytes retrieved. European journal of obstetrics and gynecology and reproductive biology. 2017; 215:50-54.
2. Zhu L. Xi Q., Zhang H., LiY., Ai J., Jin L., Blastocyst culture and cryopreservation to optimize clinical outcomes of warming cycles. Reproductive BioMedicine Online. 2013;27:154– 160.
3. Doody J. K. Cryopreservation and delayed embryo transfer–assisted reproductive technology registry and reporting implications. Fertility and sterility. 2014; 102:27-31.
4. Cobo A., Castell D., Vallejo B., Albert C., Santos J.M, Remohí J. Outcome of cryotransfer of embryos developed from vitrified oocytes: double vitrification has no impact on delivery rates. Fertility and sterility. 2013; 99:1623-1630.
5. Al-Azawi T., Tavukcuoglu S., Khaki A.A, Hasani S.A. Cryopreservation of human oocytes, zygotes, embryos and blastocysts: A comparison study between slow freezing and ultra rapid (vitrification) methods. Middle East Fertility Society Journal, 2013; 18:223-232.

6. Bedoschi G. and Kutluk O. Current approach to fertility preservation by embryo cryopreservation. Fertility and Sterility. 2013; 99(6): 1496 – 1502.
7. Arav A. Cryopreservation of oocytes and embryos. Theriogenology. 2014;81:96-102.
8. Roy T. K., Bradley C.K., Bowman M.C., McArthur S.J. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. Fertility and Sterility. 2014; 101(5):1294 – 1301.
9. Lavara R., Baselga M., Marco-Jiménez F., Vicente J.S. Embryo vitrification in rabbits: Consequences for progeny growth. Theriogenology. 2015; 84(5): 674-680.
10. Eto T., Takahashi R., Kamisako T., Hioki K., Sotomaru Y. A study on cryoprotectant solution suitable for vitrification of rat two-cell stage embryos. Cryobiology. 2014;68:147-151.
11. Oikonomou Z., Chatzimeletiou K., Sioga A., Oikonomou L. Tarlatzis B.C., Kolibianakis E. Effects of vitrification on blastomere viability and cytoskeletal integrity in mouse embryos. Zygote. 2016;1-10.
12. Diaz F., Boniolli K., Paccamonti D., Gentry G.T. Cryopreservation of day 8 equine embryos after blastocyst micromanipulation and vitrification. Theriogenology. 2016;85(5):894-903.
13. Mukaida T. and Oka C. Vitrification of oocytes, embryo, and blastocysts. Best Practices and Clinical Obstetrics and Gynaecology. 2012;26:789-803.
14. Nasrabadi, H. T.; M. Gavami,; A. Akbarzadeg,; R. Beheshti,; D. Mohammadnejad,; A. Abedelahi. Preservation of mouse ovarian tissue follicle morphology and ultra-structure after vitrifying in biotechnological protocols. Journal of Ovarian Research. 2015;8(7):1-8.
15. Araújo-Lemos P. F. B., Neto L. M. F., Melo J.V, Moura M., Oliveira M. A. L. Comparison of different cryoprotectant regimes forvitrification of ovine embryos produced in vivo. Small Ruminant Research. 2014; 119:100-106.
16. Herrid M., Vajta G., Skidmore J.A. Current status and future direction of cryopreservation of camelid embryos. Theriogenology. 2017; 89: 20-25.
17. Fesahat F., Faramarzi A., Khoradmehr A., Omidi M., Anbari F., Khalili M.A. Vitrification of mouse MII oocytes: Developmental competency using paclitaxel. Taiwanese Journal of Obstetrics and Ginecology. 2016; 55:796-800.
18. Bartolac L. K., Lowe J.L., Koustas G., Sjoblom C., Grupen C.G. A comparison of different vitrification devices and the effect of blastocoeles collapse on the cryosurvival of in vitro produced porcine embryos. Journal of reproduction and development. 2015; 61(6):525-531.
19. Roque M., Lattes K., Serra S., Sol I., Geber S., Carreras R., Checa M.A. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. Fertility and sterility. 2013; 99(1):156-162.
20. Eto T., Takahashi R., Kamisako T. Strain preservation of experimental animals: Vitrification of two-cell stage embryos for multiple mouse strains. Cryobiology. 2015; 70:150-155.
21. Caamaño J. N., Gómez E., Trigal B., Muñoz M., Carrocera S., Martín D., Díez C. Survival of vitrified in vitro–produced bovine embryos after a one-step warming in-straw cryoprotectant dilution procedure. Theriogenology. 2015; 83(5): 881-890.
22. Wong K. M., Mastenbroek S., Repping S. Cryopreservation of human embryos and its contribution to in vitro fertilization success rates. Fertility and Sterility. 2014; 102(1):19 – 26.
23. Jang T. H., Park S.C., Kim J.Y., Seok J.H., Park U.S, Choi C.W, Lee S.R., Han J. Cryopreservation and its clinical applications. Integr med res. 2017; 6:12-18.
24. Argyle C. E., Harper J.C., Davies M.C. Oocyte cryopreservation: where are we now. Human Reproduction Update. 2016;22(4):440–449.
25. Choudhary M., Soni R., Swarankar M.L., Garg S. Comparison of vitrification and slow freezing for cryopreservation of cleavage stage embryos (Day 3) and its impact on clinical outcome. International Journal of Research in Medical Sciences. 2015; 3(10):2751-2756.
26. Choi Y., Hinrichs K. Vitrification of in vitro-produced and in vivo-recovered equine blastocysts in a clinical program. Theriogenology. 2017; 87: 48-54.
27. Konc J., Kanyó K., Kriston R., Somoskyi B., Cseh S. Cryopreservation of Embryos and Oocytes in Human Assisted Reproduction. BioMed research international. 2014:1-9.
28. Gupta A., Singha J., Anzara M. Effect of cryopreservation technique and season on the survival of in vitro produced cattle embryos. Animal reproduction science. 2016; 164:162-168.